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VOL. XXVIII. No. 6] [DECEMBER, 1947

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

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on behalf of
The British Journal of Experimental Pathology. Registered Office: 120 Moorgate, London, E.C.2

Annual Subscription, January to December, post free, Two Pounds net
Single Numbers, Seven and Sixpence net

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

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Date of Publication.—The Journal is published bi-monthly, in the middle of February, April, June, August, October, and December.

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VOL. XXVIII DECEMBER, 1947 NO. 6

RELATION BETWEEN SERUM PROTEIN AND HAEMOGLOBIN IN 324 INDIAN SOLDIERS.

H. LEHMANN.

From the No. 1 Detachment, Anaemia Investigation Team, General Headquarters, India.

Received for publication September 9, 1947.

In nutritional surveys of Indian troops the impression was gained that there was more of a correlation between haemoglobin and serum protein levels than had been seen in numerous similar investigations in Europe and North America (Foy, Retter, Damkas, Depanian, Mitchell and Pitchford, 1946). Haemoglobin and serum protein were therefore measured in 324 Indian soldiers and particular attention was paid to the red cell type in cases showing a low serum protein. The investigation also gave an opportunity to test the value of the copper sulphate gravity method for serum protein determination by Phillips, Van Slyke, Dole, Emerson, Hamilton and Archibald (1945) as a means of mass detection of hypoproteinaemia.

Subjects.

The subjects were Indian sepoys stationed in the Punjab. Some of the anaemic cases were found in the anaemia wards of Military Hospitals. The ambulant soldiers were kept at rest for 30–60 min. before their blood was taken. All subjects were either fasting or within one hour after a light breakfast of sweetened tea and chapattis (unleavened bread).

Blood and Serum.

Seven to 10 ml. of blood were obtained without stasis from an arm vein using syringes previously sterilized with hot liquid paraffin. The blood was divided into two portions, 2 ml. were measured into a graduated tube containing the appropriate amount of anticcagulant (Wintrobe's dry mixture of ammonium and potassium oxalates after Whitby and Britton, 1942). This part was used for haematological analysis. The rest of the blood was slowly transferred into a test tube which was left in a tilted position for the serum to separate. The serum was withdrawn with a capillary pipette. The blood was obtained between 8 and 9 a.m., and all investigations were carried out on the same day, the only exception being the Kjeldahl estimations.

Haemoglobin.

Haemoglobin was estimated by the acid haematin method with a Sahli-Adams diluting haemoglobinometer composed of a square sectioned diluting tube and non-fading glass comparators. The instrument, including the one diluting pipette used throughout, was calibrated by the alkaline haematin method of Clegg and King (1942). Particularly in view of the criticisms of the diluting method by Macfarlane (1945) we should have preferred to use the alkaline haematin method for routine purposes as well, but we found the alkaline haematin standard solutions to fade in whatever glass-ware and under whatever conditions they were stored. To use this method successfully a photo-electric colorimeter would have been necessary which could have been standardized on one occasion for all tests with a fresh solution of alkaline haematin. However, only a visual type of colorimeter was available to us at the time.

The cell volume was measured in a Wintrobe tube, which was spun at 2500 r.p.m. for 1 hour.

Red blood cells.

Cell counts were done in a Spencer " bright line " counting chamber with an " improved Neubauer " ruling. A dilution was made in each of two calibrated pipettes, and two counts were made on each dilution. The recorded result was the average of the four counts done on the one specimen of blood.

Serum protein.

Various methods were used, but all the values recorded in Fig. 1 and 2, and in Table III were obtained by the copper sulphate gravity method of Phillips *et al.* (1945), using the formula—

Serum protein in g. per cent = 360 × (Serum Specific Gravity — 1·0070).

With plasma samples a correction was made for the added anticoagulant (Phillips *et al.*, 1945). There is now ample evidence that this method is reliable (Hoch and Marrack, 1945), and it is not intended to add yet another confirmation to those of the previous workers. However, we have made a number of comparisons of different methods on identical samples, the result of which might be of general interest.

The Kjeldahl and Nessler methods were performed following the principles laid down by the Advisory Committee on Nutrition Surveys (Bureau of Nutrition Surveys, 1945), an unpublished copy of which was made available to us at the time by the courtesy of Prof. E. J. King. In estimations done on plasma potassium oxalate only was used as the anticoagulant. Care was taken to follow the recommendations of Chibnall, Rees and Williams (1943) as regards the use of selenium dioxide as catalyst during digestion and the need for prolonged incineration.

The Biuret estimations were performed with a Lovibond Tintometer according to Harrison (1944).

Both Table I and Table II show a satisfactory agreement between the serum protein values obtained by Kjeldahl and gravity methods.

TABLE I.—*Serum Protein in 12 Samples Measured by 4 Different Methods.*

Method.	Serum protein in g. per cent. Mean.	± S.D.
Kjeldahl	7·32	1·24
Nessler	7·20	1·20
Biuret	7·33	1·38
Copper sulphate gravity	7·15	0·85

TABLE II.—*Comparison of the Copper Sulphate Gravity Method with Nessler and Kjeldahl Methods.*

Number of samples.	Type of Sample.	Serum protein in g. % mean ± S.D. Kjeldahl.	Gravity.	Number of cases showing agreement within 0·3 g. %.	within 0·4 g. %.
34	Serum	7·13 ± 1·54	7·14 ± 1·50	22 (65%)	31 (91%)
10	Plasma	7·13 ± 2·16	7·56 ± 2·58	4 (40%)	6 (60%)
		Nessler.	Gravity.		
22	Serum	7·11 ± 1·47	7·28 ± 1·22	16 (73%)	18 (82%)

It will be seen in Table II that there was a less satisfactory agreement when plasma protein was measured than was obtained with serum. We made several attempts to find an explanation for this discrepancy, but we had no success. In particular we estimated the chloride content of plasma incubated with its blood corpuscles, as we thought the gravity of the samples might have been altered by a chloride shift; no experimental evidence for this possible explanation could, however, be obtained.

Of the three determinations by gravity suggested by Phillips *et al.* (1945) the one for serum protein seems to be the most useful, both for mass investigations and for individual measurements. The haemoglobin estimation by gravity can be recommended for mass investigations, but it is not accurate enough for individual measurements (Hynes and Lehmann, 1946). Lastly, the calculation of cell volume from blood and plasma gravities cannot be recommended for either mass determinations or individual cases. This method assumes that the specific gravity of the non-haemoglobin part of the red cells is constant, and this could not be confirmed in a mass investigation on Indian soldiers (Lehmann and Kayser, 1947).

Comparison of serum protein and haemoglobin.

Fig. 1 shows the haemoglobin and serum protein values in 324 individuals. Forty-eight of these cases were definitely anaemic (haemoglobin <11 g. per cent) and would have been discovered in a search for malnourished subjects had haemoglobin estimation been the means of the mass detection. Had a serum protein value of 5·6 g. per cent and below been the criterion of malnutrition 8 cases only would have been found. 316 cases would have been passed as normal, including 40 of them with a haemoglobin of less than 11 g. per cent. 5·6 g. per cent is, according to Harrison (1944), the lower limit of the normal range of serum protein values. This figure might be considered low, but even a critical level of

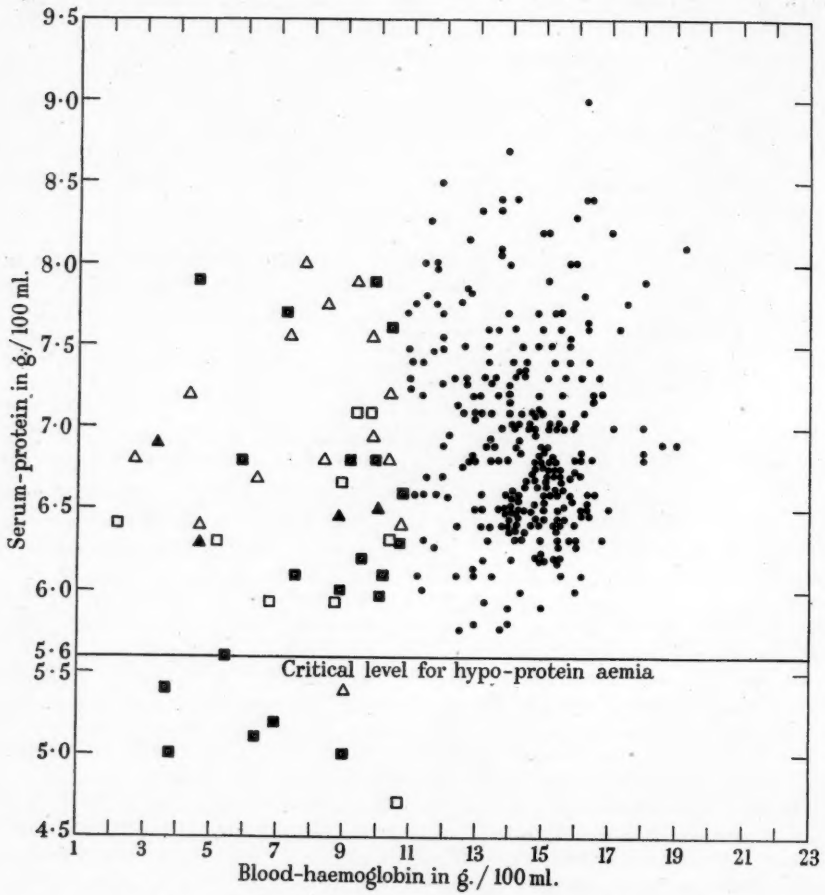

FIG. 1.—Relation between haemoglobin and serum protein in 324 Indian soldiers.

●..... Serum protein in bloods with above 10·9 g. per cent. haemoglobin.
■ □ ▲ △ ..... ,, ,, ,, ,, below 11·0 g. ,, ,,
△ Mean corpuscular volume below 100 cu.μ.
,, ,, haemoglobin concentration below 30 per cent.
▲ ,, ,, volume below 100 cu.μ.
,, ,, haemoglobin concentration above 29·9 per cent.
□ ,, ,, volume above 99·9 cu.μ.
,, ,, haemoglobin concentration below 30 per cent.
■ ,, ,, volume above 99·9 cu.μ.
,, ,, haemoglobin concentration above 29·9 per cent.

6·0 g. per cent would have eliminated not more than 12 of the 48 cases with severe anaemia and at the same time would have included 10 others, of whom 3 would have shown a haemoglobin of over 14 g. per cent. It does not seem justified to choose a yet higher critical serum protein level. Dyson (1945) found in Britain the serum protein values of 353 healthy blood donors to range from 5·65 to 7·65 g. per cent; a group of 60 individuals specially chosen for having a haemoglobin over 16 g. per cent showed serum protein values ranging between 5·91 and 7·62 g. per cent. Similarly, in 100 healthy Canadian soldiers the range of serum protein values was between 5·95 and 7·86 g. per cent. Hynes, Ishaq and Morris (1946) found the serum protein values of 70 healthy trained Indian soldiers to range between 6·3 and 7·8 g. per cent. A survey of our cases based on a critical serum protein level of 6·3 g. per cent would have detected 19 of the 48 cases of definite anaemia (< 11 g. per cent haemoglobin), but would have missed the other 29 cases in this group and in addition would have included 21 of the 187 subjects with a haemoglobin of 14 g. per cent and above.

Table III shows the percentage of cases with a serum protein < 6·4 g. per cent at various haemoglobin levels.

TABLE III.—*The Proportion of Cases with Low Serum Protein at Various Levels of Haemoglobin. 324 Cases.*

g. per cent haemoglobin.	Number of cases.	Percentage of cases with a serum protein <6·4 g. per cent.
2·0– 8·9	23	43
9·0–11·9	48	27
12·0–13·4	44	11
13·5–14·4	65	12
14·5–15·4	68	10
15·5–16·4	55	15
16·5 and above	21	5

The proportion of individuals with low serum protein is too high at normal and above normal haemoglobin levels to allow for a satisfactory correlation. Nevertheless the percentage of cases with low serum protein (under 6·4 g. per cent) can be seen in Table III to be higher in the lower haemoglobin levels and Fig. 1 demonstrates that all the cases of severe hypoproteinaemia (under 5·6 g. per cent) were found in the anaemia group. Anaemia does not necessitate a hypoproteinaemia or even a low normal serum protein, but hypoproteinaemia is only found with anaemia, and low serum protein tends to occur more frequently at the lower levels of haemoglobin. Walters, Rossiter and Lehmann (1947) have studied the pattern of recovery in malnutrition and have found that the serum protein concentration was one of the values which returned to normal at an early stage, whereas the last values to reach normal levels were body weight and haemoglobin concentration. Similarly, Fig. 1 and Table III suggest that in the development of malnutrition a fall of haemoglobin precedes that of serum protein. Haemoglobin estimation should therefore be a more sensitive test for malnutrition in its early stages than that of serum protein.

Serum protein and red cell type.

Both Fig. 1 and Table III demonstrate that there is some correlation between anaemia and low serum protein, but that the number of exceptions is so large that no statistically valid conclusions can be drawn. However, in Fig. 1 the red cell type is indicated in the cases of severe anaemia (under 11 g. per cent haemoglobin) and it can be seen that of 19 cases with a mean corpuscular volume under 100 cu.μ only one was found to be hypoproteinaemic (serum protein under 5·6 g. per cent), in contrast to a proportion of 7 out of 29 with a mean corpuscular volume above that level. Similarly only 2 out of 24 cases with hypochromic anaemia (mean corpuscular haemoglobin concentrated under 30 per cent) were hypoproteinaemic, in contrast to a proportion of 6 out of 24 cases with a mean corpuscular haemoglobin concentration of 30 per cent and above.

Fig. 2 shows in 109 cases, 48 of which had a haemoglobin below 11 g. per cent, an analysis of the red cell type as regards volume and haemoglobin concentration, and how many cases in each group had a serum protein under 6·4 g. per cent. It can be seen at a glance that the proportion of cases with low serum protein is higher in the macrocytosis group; this is particularly evident in the group with definite anaemia (haemoglobin under 11 g. per cent), but the phenomenon can also be seen in the group with a higher haemoglobin. Of all the 109 cases a low serum protein (under 6·4 g. per cent) was found in almost every second case (23 of 48) in the macrocytosis group, but only in one case out of six (10 of 61) in the group with a mean corpuscular volume under 100 cu.μ.

The macrocytic type of red cells is connected with a deficient intake of Wills factor, a type of diet which usually goes hand-in-hand with lack of valuable proteins in the food and which therefore could be expected to lower the serum protein. On the other hand, hypochromic blood cells, usually normo- or microcytic, occur in iron deficiency, a condition which might not necessarily have an influence on the protein stores of the body. Thus a diet low in valuable food factors other than iron would reduce both serum protein and haemoglobin, the red cells would be macrocytic and ortho- or hyperchromic. An iron deficiency would influence the haemoglobin formation only and the red cells would be normo- or microcytic and hypochromic. The existence of *two* factors influencing the haemoglobin level, of which only *one* would also influence the serum protein level, would explain why there is some correlation between anaemia and low serum protein, and why at the same time this correlation is erratic and has been reported as unsatisfactory in the literature and is shown as such also in the present communication. A satisfactory relationship between low haemoglobin and low serum protein levels might be expected if the cases of anaemia examined were restricted to a type caused by a diet which also lowers the serum protein, i.e. cases of macrocytic anaemia.

SUMMARY.

1. The copper sulphate gravity method for measuring serum protein was found satisfactory in the mass detection of hypoproteinaemia.

2. A survey of 324 Indian soldiers showed that haemoglobin estimation was a more sensitive test for malnutrition than the determination of serum protein.

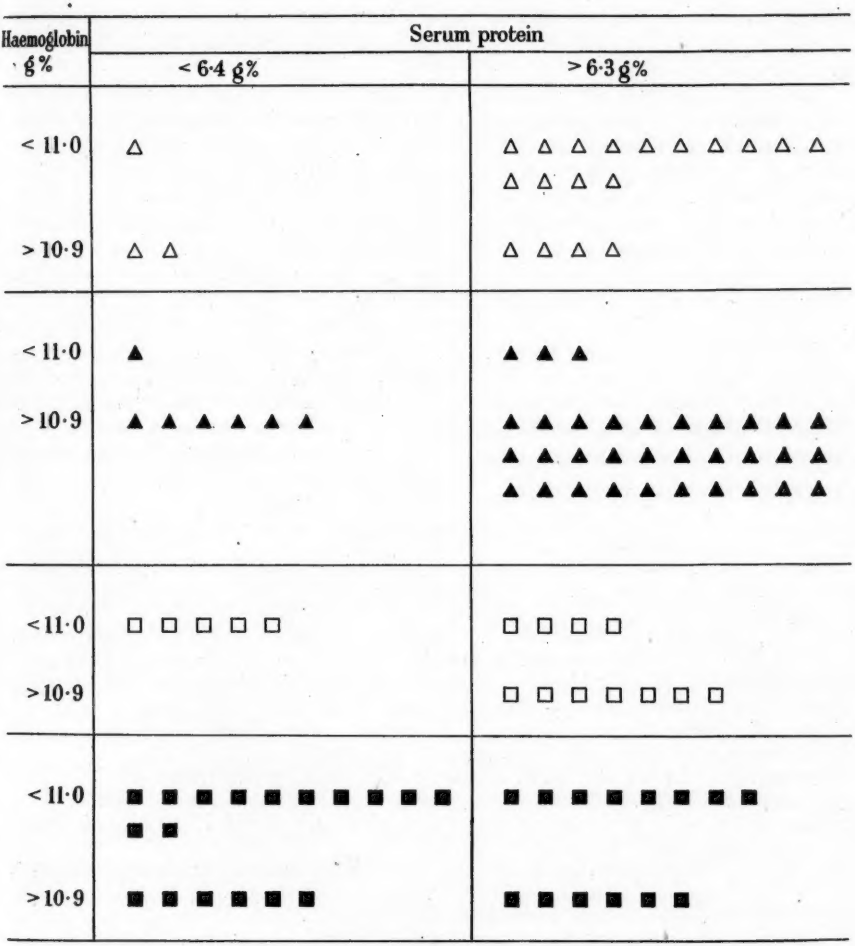

FIG. 2.—Serum protein and red cell type.

△ Mean corpuscular volume below 100 cu.μ.
,, ,, haemoglobin concentration below 30 per cent.
▲ ,, ,, volume below 100 cu.μ.
,, ,, haemoglobin concentration above 29·9 per cent.
□ ,, ,, volume above 99·9 cu.μ.
,, ,, haemoglobin concentration below 30 per cent.
■ ,, ,, volume above 99·9 cu.μ.
,, ,, haemoglobin concentration above 29·9 per cent.

3. Hypoproteinaemia was associated with anaemia, but, on the other hand, definite anaemia could be found in cases with normal or even high serum protein.

4. An attempt was made to correlate hypoproteinaemia with the macrocytic type of red cells.

5. It is suggested that the fact that there is some correlation between low serum protein and anaemia, but that this correlation is unsatisfactory, is due to the circumstance that anaemia may be caused by *two* types of dietary deficiency, of which *one* only can be expected to influence the serum protein level. Low serum protein can be expected in macrocytic, usually, but not necessarily, ortho- or hyperchromic anaemia which is found in people living on a diet lacking in vitamins and valuable protein. Hypochromic, normo- or microcytic anaemia occurs when iron is the limiting factor in the diet, a condition which should not necessarily lower the protein stores of the body.

My thanks are due to the D.M.S. in India for permission so publish this paper; Prof. E. J. King and Dr. Lucy Wills for their advice; Lieut.-Col. Puri, Lieut.-Col. Taneja and Major Hynes for their support. I was helped in the Kjeldahl estimations by Sergeant J. W. Simpson, and in the procurement of the specimens by Sergeant F. P. Kayser. For this and much other help I should like to render them my thanks.

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THE DEVELOPMENT OF ANTHRAX FOLLOWING THE ADMINISTRATION OF SPORES BY INHALATION.

J. M. BARNES.

From the Microbiological Research Department, Experimental Station, Porton.

Received for publication October 6, 1947.

ALTHOUGH anthrax is one of the oldest recognized bacterial diseases, little or nothing is known about the pathogenesis of this infection. Nothing comparable to the work of Fildes on the factors determining the germination of tetanus spores in the tissues has been done in the case of anthrax.

Pulmonary anthrax was produced experimentally by Buechner (1886). He infected rabbits, guinea-pigs and mice by exposing them to clouds of anthrax spores put up in small chambers. He showed that the A.L.D. by inhalation was considerably smaller than by ingestion. He cut sections of the lungs of animals killed at different intervals after exposure in an attempt to find the site at which germination of the spores and multiplication of the resulting vegetative forms took place. His paper records the difficulties encountered. In only one section did he find a small aggregation of vegetative forms in the wall of an alveolus. Similar experiments were carried out by Ferguson (1945). He exposed mice to airborne anthrax spores and killed them at a time calculated from earlier experiments to be within a few hours of the onset of the terminal septicaemia. A careful search of many sections revealed only two small foci in one section in which a few bacilli were visible in a recent haemorrhage in the alveolar wall.

The experiments to be described were carried out in an attempt to throw some more light on the pathogenesis of anthrax spore infections and the experimental pulmonary type of infection was chosen. This experimental disease may bear little relation to the now rare occupational disease known as wool sorters' disease. Fraenkel (1925) examined the specimens from a number of these cases, and was able to demonstrate a lesion similar to the typical " pustule " in the walls of the trachea or bronchus. Such lesions are not seen in the experimental infection to be described.

It should be pointed out that the work described here represents elaboration of the fundamental experiments carried out earlier by Dr. Henderson and his colleagues, and which have not yet been published. They first produced this type of infection and devised the apparatus whereby accurately estimated doses of the spores could be administered under safe experimental conditions.

METHODS.

(a) *Production of spore clouds.*—This was carried out in the apparatus designed by Henderson (1943). The animals were exposed to a passing cloud which consisted of 98 per cent single " dry " anthrax spores. The spores were put up

from suspensions in water; and were virtually dry by the time they reached the point at which the animals were exposed.

The spores were taken from a fully lysed stock suspension of a concentration of about 4×10^{10} per ml. This stock was of proven virulence, and neither this nor the concentration altered appreciably during the course of the experiments.

(b) *Exposure of the animals.*—Rabbits, guinea-pigs and mice were used and were of mixed stock, but showed no evidence of varying in their natural susceptibility to the infection. During the exposure to the spores the animals were held in such a way that only their noses projected into the passing cloud. They were exposed for periods varying from 1 to 5 minutes and no anaesthesia was employed.

(c) *Calculation of dose.*—The dose was always expressed as the number of spores calculated to have been taken in by the animal with the inspired air during the period of exposure. The concentration of the spores in the cloud was measured by passing the cloud through a capillary tube acting as a criticial orifice and collecting the impinged spores in distilled water. Counts were made on appropriately diluted samples of the water. The duration of exposure was known, and the respiratory intake of the particular species was taken from the nomogram prepared by Gaddum (1944).

Constant and repeatable results were obtained in experiments extending over a period of more than two years, so that clouds of spores of the desired concentration could be put up by choosing a suitable dilution for the stock spore suspension before spraying it.

(d) *Detection of anthrax spores in the tissues of exposed animals.*—Quantitative estimations of the number of spores in the lungs, stomach and lymph glands of exposed animals were made by making bacterial counts on homogenized suspensions of the organs. These suspensions were prepared by grinding the tissues in distilled water with an electrically driven glass homogenizer. The presence or absence of spores in different tissues was determined by culturing fragments in tryptic digest of meat broth for 48 hours.

(e) *Bacterial counts.*—The method of counting the spore suspensions was essentially that described by Miles and Misra (1938). Standard drops of a suitably diluted suspension were placed on the surface of tryptic meat or 1 per cent Evans peptone agar and incubated for either 10 hours at 37° C. or 14 hours at 30° C. For anthrax spores plate counts could be rapidly and accurately made when the colonies did not exceed 200 per plate. For any estimation of spore concentrations a total of at least 400 colonies was counted.

RESULTS.

1. *The lethal dose of anthrax spores by inhalation.*

Rabbits, guinea-pigs and mice were almost uniformly susceptible to infection by a very small number of anthrax spores of the strain employed in these experiments when administered by subcutaneous injection. A local lesion developed, and was invariably followed by a fatal generalized infection. On the other hand, it was found that when administered by inhalation far larger numbers of spores were needed to produce a uniformly fatal infection. In Table I the lethal doses by injection and inhalation are compared in the case of the rabbit, guinea-

pig and mouse. It should be emphasized here that the dose by inhalation is expressed as the number of spores calculated to have been inhaled, and this is undoubtedly greater than the number actually retained by the animal (*vide infra*).

TABLE I.—*A Comparison of the Lethal Dose of Anthrax Spores by Inhalation and by Subcutaneous Injection in a Number of Susceptible Animal Species.*

		Subcutaneous.	Inhalation.
Rabbit	2 kg.	100–1000	600,000
Guinea-pig	350 g.	100–1000	370,000
Mouse	20 g.	10–100	1,400,000

2. *The course of the experimental pulmonary infection.*

Mice, guinea-pigs and rabbits exposed to a lethal dose of anthrax spores by inhalation die within 7 days of the exposure. Animals that survive exposure to an average lethal dose for periods of 7 days apparently do so indefinitely under the normal conditions of captivity.

The exposed animals appear to be quite healthy until within a few hours of death, when they collapse. They do not exhibit any symptoms characteristic of a pulmonary disorder. Death usually takes place from the 2nd to 4th day after exposure. At post-mortem the picture is that characteristic of acute anthrax septicaemia following the injection of spores under the skin. There may be a varying amount of oedema of the mediastinum, but there are no specific pulmonary lesions, either naked eye or microscopic.

3. *The number of inhaled spores retained in the lungs of exposed animals.*

In view of the great discrepancy between the size of the lethal dose of anthrax spores by injection as compared to that by inhalation, it seemed important to determine the actual number of the inhaled spores that remained in the lungs of animals exposed to clouds of single " dry " spores.

The number of spores present in the lungs of mice and guinea-pigs was determined for groups of these animals killed both immediately and at different intervals after exposure to known concentrations of anthrax spores. In Table II the results of a number of experiments are summarized, and the figure for the numbers of sporse found in the lungs of these animals is expressed as a percentage of the numbers calculated to have been inhaled with the inspired air. It will be seen that initially only some 20–25 per cent of the inhlaed spores are to be recovered from the lungs, and that this number gradually diminishes over the ensuing 24 hours. Very similar figures were obtained when animals were exposed to clouds of *B. subtilis* spores put up in exactly the same way. Both the percentage initially retained and the rate of their disappearance during the first 24 hours after exposure were the same.

It seemed probable that large numbers of the spores would have been taken out of both the inspired and expired air during its passage through the upper respiratory passages. No direct estimations of the numbers in the nose, etc., were made, but counts were made of the spores in the stomach of exposed mice. Counts were made on the stomachs and lungs of the same groups of mice killed

TABLE II.—*The Number of Spores (expressed as a Percentage of the Number calculated to have been Inhaled) Present in the Lungs immediately and at Intervals after Exposure to Airborne Clouds of Single Anthrax Spores.*

	Hours after exposure.	Percentage of inhaled spores in lungs. Experiment				
		1	2	3	4	5
Mice (groups of 10)	0	19·7	18·3			
	½	16·4	14·0			
	1	13·8	16·0			
	2	15·8	11·4			
	4	13·0	10·3			
	6	15·2	5·5			
	24	13·5	5·4			
Guinea-pigs (groups of 2 or 3)	0	25·8	25·8	27·1	24·8	30·2
	1	..	24·3			
	2	21·4	16·0	..	23·0	25·5
	4	18·7	18·8	26·8	21·0	17·2
	6	14·5	14·2			
	24	12·9	6·2	16·7	11·6	10·0

at different intervals after exposure, and showed that within ½–1 hour after exposure, up to 70 per cent of the number of spores calculated to have been inhaled could be isolated from the stomach. The numbers in the stomach rapidly fall, due either to their destruction in, or passage down, the alimentary tract (Table III). Again, *B. subtilis* spores behave in exactly the same way as anthrax spores.

TABLE III.—*Percentage of Inhaled Anthrax Spores Recovered from the Stomach of Exposed Mice Killed at Different Intervals after Exposure.*

	Hours after exposure.	Percentage of inhaled spores in stomach. Experiment	
		1	2
Mice (groups of 10)	0	21·0	8·2
	½	49·0	34·0
	1	68·0	40·5
	2	14·0	14·3
	4	3·5	6·0
	6	0·8	2·7

4. *The spread of inhaled anthrax spores from the respiratory tract.*

The cervical lymph glands were removed from guinea-pigs killed 4 and 24 hours after exposure to anthrax spore clouds and homogenized suspensions were cultured. Despite the large numbers of spores retained in the upper respiratory tract, only a very occasional spore could be isolated from the draining lymph glands.

The tracheo-bronchial group of lymph glands were examined in the same way. Small and variable numbers of spores could be found in these lymph glands in animals killed 24 hours after exposure. Twenty-six guinea-pigs thus examined had from 0–254 spores (average 70) 24 hours after exposure to a cloud of anthrax spores that had resulted in an average of 1 million spores being retained initially in the lungs. During this period of 24 hours an average of 500,000 spores had disappeared from the lungs. These findings suggest that either—

(*a*) The spores are destroyed within the lungs.

(*b*) They leave the lungs by routes other than the lymphatics.

(*c*) They enter the lymph glands and are rapidly destroyed with or without prior germination.

(*d*) They enter the lymph glands and rapidly pass on, presumably into the blood stream.

No evidence could be obtained of the direct entry of inhaled anthrax spores into the blood stream. Cultures were made from the blood, spleen and liver of a series of rabbits killed at different intervals up to 48 hours after exposure to a lethal dose of anthrax spores. No single positive culture was obtained. That anthrax spores can be cultured from these organs was shown in other experiments where it was found that after the *intravenous* injection of only 50,000 anthrax spores, cultures from the spleen would be positive for at least 3 weeks in surviving animals.

5. *The effect of "Tergitol" on inhalation anthrax.*

Young and Zelle (1946) reported that the virulence of inhaled anthrax spores could be enhanced by spraying the spores from solutions of a commercial detergent named "Tergitol," As this increase in virulence was quite striking, and because the "Tergitol" alone was not obviously injurious to the lungs, this observation was repeated and pursued in the hope that the mode of action of the "Tergitol" might be elucidated, and so throw some light on the pathogenesis of the infection itself. Experiments were carried out with samples of "Tergitol" supplied by the maker's agency in this country.* The effect on virulence is readily observed by spraying the spores from suspensions made up in the appropriate dilution of "Tergitol" in water. By the use of such suspensions the following observations were made :—

(a) *The effect on death rate of exposed animals.*—Animals were exposed to anthrax spore clouds put up from suspensions in water, and compared to groups of similar animals exposed to clouds from suspensions in different concentrations of "Tergitol" in water. The results of three such comparative experiments are presented in Table IV. Sprayed from a solution of 5 per cent "Tergitol" anthrax spores have their virulence for guinea-pigs enhanced some 16 times. A similar effect was observed for mice. At post-mortem there was no distinction to be made between the two groups of animals. There was no evidence of any irritant effect of the inhaled "Tergitol" on the lungs. That this enhancement of virulence

* Samples of "Tergitol" 4 were kindly supplied by the manufacturers, General Metallurgical and Chemical Corporation, Holborn, London. They stated that the formula of the material supplied was as follows :

$$\begin{matrix} C_4H_9.CH.C_2H_5.CH_2CH_2 \\ (CH_3)_2.CH.CH_2 \end{matrix} \!\!>\!\! —CHO.SO_3Na \quad . \qquad 25 \text{ per cent in water.}$$

TABLE IV.—*Comparison of the Virulence of Inhaled Anthrax Spores Sprayed from* (a) *Water*; (b) "*Tergitol.*"

Experiment.	Spores suspended in—	Cloud concs. spores/litre.	Deaths/total exposed.	Ratio of potency.
1	*a.* Water	$7{\cdot}5 \times 10^5$	1/20	2·05 (1·8—2·03)
	b. 0·1% "Tergitol"	$7{\cdot}2 \times 10^5$	3/20	
2	*a.* Water	$6{\cdot}56 \times 10^5$	6/20	
	b. 1·0% "Tergitol"	$6{\cdot}67 \times 10^5$	17/20	5·18 (2·5—10·5)
3	*a.* Water	$7{\cdot}05 \times 10^5$	2/20	
	b. 5·0% "Tergitol"	$7{\cdot}62 \times 10^5$	19/20	16·4 (37·1—7·2)

does not depend on some direct effect of the "Tergitol" on the lungs was clearly shown in experiments, where animals were exposed to clouds of spores and "Tergitol" put up separately and mixed before reaching the animals. The amount of "Tergitol" inhaled would be exactly the same, but where the spores had been put up from a suspension in "Tergitol" one may assume that a layer of the substance is formed round the spore as it loses its surrounding droplet of suspending fluid.

Even when the animals were exposed to mixed clouds of anthrax spores from water and *B. subtilis* spores from "Tergitol" there was no enhancement of the virulence of the anthrax, though it could be assumed that under such conditions exactly the same amount of "Tergitol" would have been carried into the same parts of the lungs on the *B. subtilis* spores as it would have been on the anthrax spores.

(b) *The effect of "Tergitol" on the numbers of anthrax spores retained in the lungs.*—It seemed to be possible that "Tergitol" might exert its effect on virulence by increasing the number of inhaled spores that reached and remained in the lungs. Estimations were therefore made on mice and guinea-pigs exposed to clouds of anthrax spores put up from 5 per cent "Tergitol" in water. These showed that smaller rather than greater numbers of inhaled spores could be found in the lungs of animals exposed to clouds from "Tergitol."

The reduction in the number of inhaled spores found in the lungs was greater in the mice, falling from an average of 19·5 per cent when the spores were sprayed from water to 3·1 per cent when they were sprayed from "Tergitol." In the case of the guinea-pig the average percentage retained was reduced from 26·7 to 19·8.

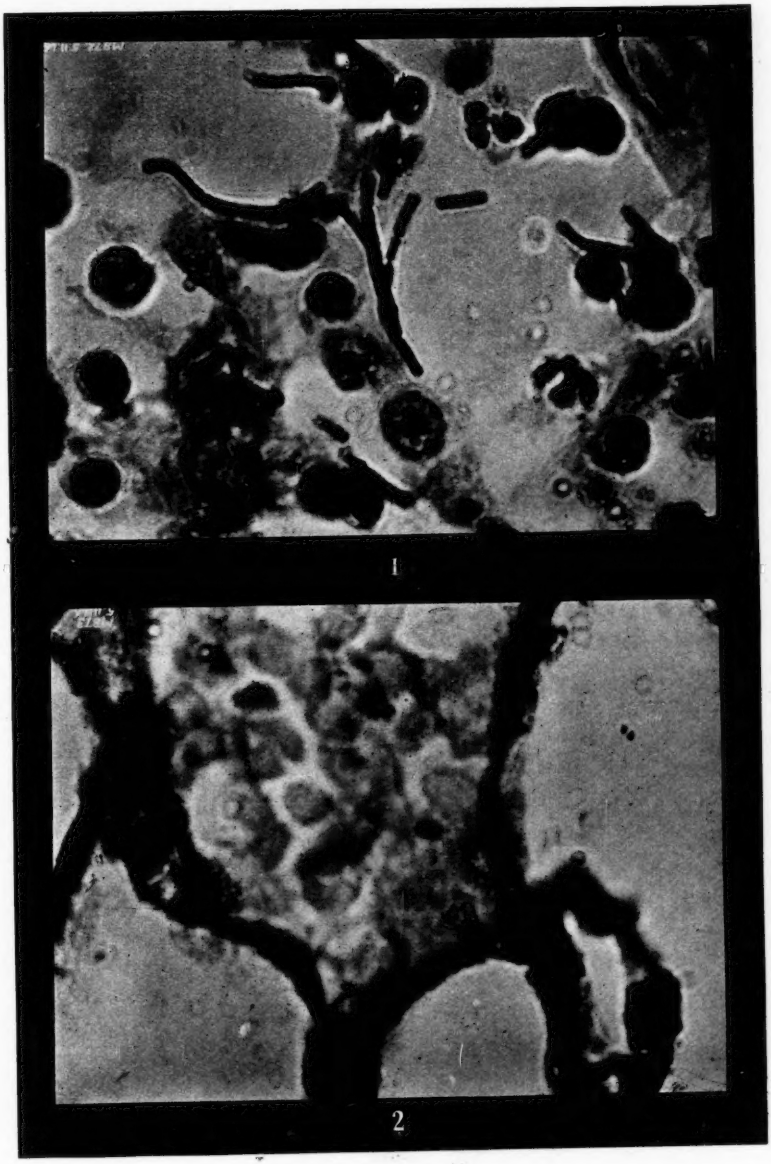
On the other hand, the percentage of inhaled spores recovered from the stomach of the mice was the same after spraying from water and from "Tergitol." This suggests that in some way the "Tergitol" increases the number of spores retained in the upper respiratory tract.

(c) *The effect of "Tergitol" on the number of inhaled spores reaching the bronchial lymph glands.*—Examination of the tracheo-bronchial lymph glands of guinea-pigs 24 hours after exposure to anthrax spore clouds put up from "Tergitol" showed

DESCRIPTION OF PLATE.

FIG. 1.—Periphery of a bronchial lymph gland taken from a guinea-pig killed 24 hours after exposure to 3 A.L.D. of anthrax spores suspended in 5 per cent "Tergitol" (Gram stain).

FIG. 2.—Part of the lung of the same animal showing a blood vessel devoid of organisms.



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that on the average about twice as many spores could be found there than after exposure to clouds put up from water. This difference is enhanced in significance by the fact that the number of spores reaching the lungs after spraying from "Tergitol" was about 20 per cent less than after spraying from water. On the other hand, the "Tergitol" spores disappear more rapidly from the lungs during the first 24 hours, and once again the numbers in the glands (average 172) bear little relation to the numbers that have disappeared from the lungs in the same period (average 700,000).

(d) *The detection of early germination of anthrax spores after exposure to clouds from "Tergitol."*—When the tracheo-bronchial lymph glands of a number of guinea-pigs killed 24 hours after exposure to three times an average lethal dose of anthrax spores sprayed from "Tergitol" were cultured, they were often found to yield a confluent growth of anthrax instead of an occasional colony. Examination of smears of the suspensions showed the presence of vegetative forms. Counts made at the same time from homogenized suspensions of the lungs revealed *no increase* in the expected number of colonies.

Further experiments then showed quite definitely that after exposure to clouds of anthrax spores from "Tergitol," multiplication of vegetative forms could be clearly demonstrated in the tracheo-bronchial lymph glands before there was any evidence of germination and multiplication in the lungs themselves and while the spleen and blood cultures were still sterile. Fig. 1 shows the numbers of vegetative forms in the peripheral sinus of a bronchial lymph gland of a guinea-pig at a time when the blood culture was sterile and no vegetative forms could be seen in the lungs (Fig. 2).

Further experiments were then carried out to try to discover whether the same phenomenon took place after the inhalation of anthrax spores sprayed from water. Unfortunately after a similar dose of spores, the time of death of the exposed animals falls over a much wider range when the suspension is in water than when it is in "Tergitol." As a result it is more difficult to kill the animals at a time calculated just to precede the general invasion of the blood stream.

A group of 20 guinea-pigs were exposed to three times an average lethal dose of spores put up from water, and groups were killed at different intervals from 36–48 hours after exposure. Counts were made of the lungs, bronchial lymph glands and spleen with the following results:

Number of animals.	Counts of the unheated suspensions of—			Conclusion.
	Lung.	Glands.	Spleen.	
5	Normal	*Nil*	*Nil*	No infection yet developing.
4	,,	Raised	,,	Multiplication started in glands.
3	,,	Massive	Few colonies	Early invasion of blood.
6	Raised	,,	Massive	Early generalized infection.
2	Massive	,,	,,	Fully developed septicaemia.
20				

These results suggest that after the inhalation of anthrax spores germination and multiplication of the resulting vegetative forms first takes place in the lymph glands, and that the subsequent generalized infection follows spread from this initial focus.

DISCUSSION.

This experimental inhalation anthrax is not a true pulmonary disease, but only an example of the introduction into the body of virulent organisms through the undamaged lungs with the subsequent development of a characteristic general infection. The interesting fact is that the fatal dose by this route is so much greater than by injection of the spores under the skin. Evidence obtained during the carrying out of the spore counts on the lungs of exposed animals suggests that the anthrax spores find the lungs a very unfavourable medium in which to start to germinate. When anthrax spores are placed in a rich medium such as tryptic digest broth even at room temperature, the first stage of germination take place almost instantaneously, and the spores become heat sensitive and are killed by exposure to temperatures of 65° C. In diluted medium the same effect takes place, but the speed with which it does so is increased by incubating at 37° C. On the other hand, spores may be removed from the lungs of guinea-pigs several days after exposure, and after they have been lying in the pulmonary tissue at 37° C. and yet they are still heat-resistant. On the other hand, if these spores are left at room temperature for an hour in the presence of the homogenized lung tissue in which they are being counted, the greater number will become heat sensitive. In other words a factor is missing in the whole lungs which is released when the tissue is damaged. It is possible that the release of this factor may be important in determining the smaller dose of spore necessary to produce infection when introduced by injection.

The evidence in this paper suggests that the lymph glands are a favourable site for the germination of anthrax spores. It is not possible to say whether the inhaled spores that disappear from the lungs are destroyed *in situ*, or whether they migrate to the lymph glands and are destroyed there with or without germination. They certainly do not accumulate in the glands, nor is there any evidence that they pass through the glands to enter the blood stream. The spores may reach the glands by direct spread along the lymphatics or within phagocytes. Either route seems to be a possible one in the case of inert dust particles (Arnold, 1885). That spores are readily taken up by the polymorphs can be shown after injection into the skin of rabbits. Within a few hours polymorphs migrate to the spot and can be seen loaded with spores. This has been observed after the injection of very concentrated suspensions (0·1 ml. of 2×10^{10} spores per ml.).

How " Tergitol " exerts its effect remains completely unknown. It obviously has to be in some intimate relationship to the anthrax spore, and apparently in some small way it increases the rate of the removal of the spores from the lungs to the glands. In the glands it may conceivably act as a growth factor for the emerging vegetative forms, or alternatively interfere in some way with the normal mechanism in the glands which destroy the greater number of the spores.

This property of enhancing the virulence of inhaled anthrax spores is shared

by a number of other surface active agents and also the polymer "Solvar."* It has not been possible to carry out exhaustive tests in order to define the chemical or physico-chemical factors necessary for the production of this effect. It is obvious, however, that chemical substances which are inhaled with particles may considerably influence their behaviour after they reach the interstices of the lungs.

SUMMARY.

1. Susceptible laboratory animals have been infected with anthrax by means of inhaled dry spores. A brief description is given of the characteristics of the infection and the means whereby it was produced.

2. This experimental infection is not a true pulmonary disease, but serves to show how infective particles may set up a generalized infection after introduction through the uninjured respiratory tract.

3. Much higher minimum lethal doses of anthrax spores were necessary to produce infection by inhalation than by subcutaneous injection.

4. Quantitative determinations revealed that approximately 20 per cent of the total number of spores calculated to have been taken in with the inspired air remained behind in the lungs. Up to 70 per cent of the number inhaled could be recovered from the stomach half an hour after exposure. These spores had probably travelled from the upper respiratory tract.

5. No evidence was obtained that inhaled anthrax spores invaded the blood stream directly. Very few spores could be found in the lymph glands draining lungs 24 hours after exposure, although during that same period about half those that had been in the lungs immediately after exposure had disappeared. The spores of the avirulent *B. subtilis* behave in exactly the same way as the virulent anthrax spores—at least until the process of germination begins.

6. The mortality among animals exposed to anthrax spores sprayed from solutions of the detergent "Tergitol" may be raised tenfold. "Tergitol" does not exert its effect by any direct action or injury to the lung tissue. Its properties are shared by a number of other non-irritant chemical substances.

7. After exposure to spores sprayed from "Tergitol" animals show a greater number of spores in the tracheo-bronchial lymph glands 24 hours later. It was also shown that infection begins by germination and multiplication of the vegetative forms in the bronchial lymph glands. This may have proceeded to a considerable extent before the blood stream is invaded. The spores in the lungs show no evidence of germination.

* The following compounds were tested and found to enhance the virulence of inhaled anthrax spores:

SAPONIN. Commercial. B.D.H.
AEROSOL O.S. Alkyl-aryl-sulphonate. American Cyanamide & Chem. Corp.
AEROSOL O.T. Di-octyl ester of sodium sulpho-succinic acid. Ditto.
CETAVLON. Cetyl-trimethyl-amonium-bromide. I.C.I.
TERGITOL 7. Sodium salt of a higher secondary alcohol. General Metallurgical Corp.
SOLVAR. Polyvinyl alcohol. Shawinigan Chemical Corp., Canada.
BILE, OX. Commercial. B.D.H.

The following were tried but showed no effect.

DISPERSOL. O.G. Undisclosed. I.C.I.
MUCIN. Gastric. Wilson Laboratories, Chicago.
TEEPOL, X. Sulphated secondary alcohol. Technical Products, Ltd., London.

8. The action of "Tergitol" remains obscure. It may be a growth factor, or it may in some way inhibit the natural defences of the tissues. It exerts its action without producing any demonstrable tissue change.

I wish to thank Dr. D. W. Henderson, Chief Superintendent, Microbiological Research Dept., for his interest and invaluable assistance during the course of the work and in the preparation of the paper. This is published by permission of the Chief Scientist, Ministry of Supply.

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VAGARIES OF THE AGGLUTINATION-INHIBITION REACTION WITH NEWLY-ISOLATED STRAINS OF INFLUENZA VIRUS.

C. H. STUART-HARRIS AND M. H. MILLER.

From the Department of Medicine, University of Sheffield.

Received for publication October 10, 1947.

IN the early part of January, 1947, a number of explosive outbreaks of influenza occurred among German prisoners-of-war in camps in the Midlands. Study of garglings and sera from patients in three different camps resulted in the recovery of four strains of an influenza virus, and antibody responses directed against influenza virus A were also detected. This paper is concerned with an account of certain peculiarities of the new virus strains, particularly in regard to the antibody response exhibited by the human cases of influenza. A full explanation of these peculiarities has not been obtained, but the results are recorded because of their importance to those engaged in work involving use of the agglutination-inhibition test.

METHODS.

Garglings were obtained from patients as soon as possible after the onset of illness and were collected in 20 c.c. of sterile saline, to which broth or glycerine was added before storage in an ordinary refrigerator. Prior to test, penicillin and streptomycin were added to the garglings to a final concentration of 500 units

of each antibiotic per c.c., and 0·2 c.c. of the mixture were then inoculated into the amniotic sacs of 13-day-old developing chick embryos, incubated prior to inoculation at a temperature of 38–38·5° C. After inoculation the embryos were incubated at 37° C. and examined in 3 or 4 days' time. Amniotic and allantoic fluids were then harvested after chilling of the eggs, and were examined for agglutinins by titration and mixture of equal volumes of dilutions of fluids in saline with 1 per cent suspensions of guinea-pig or fowl red blood cells. The deposit was examined after settling had occurred. Passage was carried out by amniotic inoculation of further 13-day-old embryos using dilutions of chick lung emulsion in amniotic fluid. In the case of negative results, " blind " passage was continued for between two and six generations of embryos.

Sera were tested by Salk's modification of the red cell agglutination-inhibition test of Hirst (Salk, 1944). Allantoic fluid of the standard laboratory strain PR8 was used after reconstitution of a dried culture received from the National Institute for Medical Research. Allantoic fluids from eggs infected by us with this or other strains were also used in the serum tests, in which a final concentration of 4 minimal agglutinating doses of antigen was employed in each tube containing the serum dilution. A final concentration of 0·125 per cent of fowl red cell suspension prepared by centrifugation of citrated blood from individual fowls without pooling was used both in titration of antigen and in serum-inhibition tests. The latter were performed on sera from ferrets convalescent from infection with standard or recently-isolated strains of virus, from a horse hyperimmunized with PR8 mouse-lung virus, and on pairs of human sera collected from patients during the acute and convalescent stages of illness. All sera were inactivated at 56° C. for 20 minutes before use.

Neutralization tests in mice were performed in the usual way, employing intranasal inoculation of groups of 4 mice. One of the new strains of virus was adapted to mice for the purpose of these tests.

Recovery of the new strains.

The four virus strains recovered from the three outbreaks of influenza resulted from inoculation of batches of eggs with each of five garglings, which were tested after one or two days storage, in an ordinary refrigerator. One of these garglings and two others stored for six weeks in the refrigerator failed to infect chick embryos. The four strains differed only slightly in their behaviour on initial isolation. In general the amniotic fluids from the first generation of eggs gave no agglutination of fowl red cells, but agglutination was sometimes obtained with guinea-pig cells at dilutions beyond the range of the haemagglutinin present in normal chick amniotic fluid (Commission on A.R.D., 1946). Table I shows the ratio fowl/guinea-pig agglutinin titres in individual fluids in the first and subsequent generations of eggs. A negative result with a 1 : 10 dilution of fluid is recorded as 0. Figures in brackets were obtained with allantoic fluids. These results were interpreted as indicating a modification of the red cell agglutinin of human influenza virus occurring on adaptation to the egg and referred to by Burnet as the O-D variation (Burnet and Bull, 1943 ; Burnet and Stone, 1945). One of the four strains (Woiteki) remained in the O phase somewhat longer than the others. Most work was carried out with the Kunz virus, which gave good fowl haemagglutinin titres in allantoic fluids by the 3rd egg generation.

TABLE I.—*Agglutination of Fowl and Guinea-pig Red Cells by Amniotic and Allantoic Fluids.*

	1st generation.	2nd generation.	3rd generation.
Baumann*	?/40 0/?	0/0 0/10 0/20 0/0 0/160	640/320 320/640 40/160 <20/160
Woiteki*	0/0 0/0	0/0 0/20 0/40 (10/80) 80/320	10/80 0/0 10/80 40/80
Kunz	0/80 20/320 0/10	160/320 0/10 0/0 0/0 (40/80) (40/40) (160/80)	640/320 640/640 (160/160) (320/80)
Kuhlmann	0/0 0/0 0/80 0/0	160/160 80/80 (80/80) (80/80)	320/? (2560/?) (1280/?) (1280/?)

Each fraction represents Fowl/guinea-pig agglutinin ratio of an individual egg fluid. ?=Test not done. Brackets represent results on allantoic fluids; the unbracketed results were obtained on amniotic fluids.
* Recovered from the same outbreak.

Agglutination-inhibition tests.

As soon as possible after establishment of the Kunz strain in eggs, when fowl haemagglutinins were present in allantoic fluids to a titre of 1/320 or more, inhibition tests were performed with available immune animal sera in order to establish the antigenic character of the strain. The results were surprising, because neither the hyperimmune horse PR8 serum nor the convalescent Lee ferret serum gave good titres of inhibition of agglutination by the virus. Further sera were therefore obtained from Dr. J. A. Dudgeon of the National Institute for Medical Research, and the Kunz virus was sent to Hampstead for preparation of a convalescent ferret serum. Tests with these sera confirmed the previous ones, and indicated that sera from ferrets convalescent from infection with viruses recovered from recent outbreaks in the south of England (Dudgeon *et al.*, 1947) or from the Kunz strain gave much higher titres of inhibition of the Kunz virus strain than standard anti-A sera prepared from the PR8 virus. The tests were extended to the other virus strains recovered by us, and to pairs of sera collected in the acute and convalescent stages of cases of human influenza. Table II summarizes a number of results with animal and human sera and with the four new strains of virus. The human sera were collected from the same patients as those whose garglings had yielded the virus strains. The titres obtained with convalescent human serum against "homologous" virus were much lower than those customarily encountered, and considerably lower than those obtained with convalescent ferret serum. In three instances also the rise of antibody titre in the convalescent serum compared with the acute specimen from the same patient was trivial. The table also shows the titres obtained with the same sera tested against PR8 virus. Better rises of antibody against PR8 than against homologous virus were demonstrated in three of the four cases, although the ferret serum from the convalescent Kunz ferret had a lower titre of inhibition against PR8 than against Kunz virus. Another source of confusion was the result obtained by testing the various strains with serum from a ferret convalescent from swine influenza virus (Shope). This serum gave as high a titre of inhibition of the new virus strains as did the PR8 ferret serum—a fact which suggested the possibility that the new strains were more closely related to swine influenza virus than to human influenza virus A. Such a relationship would not, however, explain

TABLE II.—*Agglutination-Inhibition Tests with Newly-isolated Strains and with PR8 Virus.*

Serum.	Virus antigens. Baumann.	Kuhlmann.	Kunz.	Woiteki.	PR8.
(*a*) Animal.					
PR8 ferret serum	160	320	320	80	**5120**
Kunz ,, ,,	1280	1280	**2560**	320	160
Shope ,, ,,	320	320	320	..	<40
Lee ,, ,,	..	<20	<16	<20	<20
(*b*) Human.					
Baumann 1 (acute)	**80**	80	80	..	80
,, 2 (convalescent)	**160**	160	160	..	640
Kulhmann 1	20	**20**	20	..	80
,, 2	80	**80**	80	..	160
Kunz 1	160	..	**80**	..	160
,, 2	320	..	**160**	..	640
Woiteki 1	80	80	80	**80**	80
,, 4	80	160	160	**80**	160

the contrast between the inhibition titres obtained with the human convalescent sera against their homologous virus strains and against PR8 virus. Moreover, tests with PR8 virus or sera from other cases of influenza from the same outbreaks as those yielding the new virus strains gave the results ordinarily obtained in influenza A. Similar results were also obtained by Hoyle and Fairbrother (1947) and by the workers at the National Institute for Medical Research (Dudgeon *et al.*, 1947) with the viruses recovered by them and with the sera from outbreaks of influenza in the South of England. It seemed probable that the inhibition tests with the newly-isolated viruses were in some way unsatisfactory, and failed to reflect the antibody content of the actual sera. We therefore investigated the three variable factors concerned in the test, the virus antigen, the red cell, and the serum, in the hope of throwing some light on the anomalous results.

(a) *The virus antigen.*

Tests recorded above were performed with the new strains after relatively few egg passages (3rd to 5th egg generations). It seemed possible that the O-D variation might be concerned with the anomalous results, and that the antigens which were used had been prepared from virus in an unstable phase. The Kunz virus was therefore passaged repeatedly by the amniotic route. A comparison of serum inhibitory titres obtained with allantoic fluid from the 10th generation of eggs with titres obtained with fluid from the 3rd generation showed no significant differences.

At a later date a suggestion was received from Dr. Thomas Francis, who had also encountered difficulties in the inhibition test with strains of virus recovered in the U.S.A. in 1947. Dr. Francis considered that a better antigen for the inhibition test might be obtained by adapting to mice the virus recovered originally in the egg and then re-inoculating eggs with the mouse lung virus. The Kunz virus had already by this time been adapted to mice in order to perform neutralization tests in mice. Antigen for the inhibition test was therefore prepared by inoculation of Kunz mouse lung filtrate into eggs. The results obtained with this "mouse-egg" strain in tests of pairs of human sera differed slightly from those obtained with the original egg strain. In general the acute sample of serum gave lower titres of inhibition with the mouse-egg strain, whereas the convalescent serum gave either the same or lower titres. Thus the difference in inhibitory titre between the acute and convalescent serum from the individual patient was greater with the "mouse-egg" strain than with the "egg" strain in a few instances, but there were more instances in which the two virus strains gave similar differences between the members of a pair of human sera. Furthermore, the "mouse-egg" strain was not inhibited by convalescent human serum more readily than the "egg" strain, and it did not therefore give inhibitory titres of the order exhibited by the PR8 virus.

Thus no indication was given by these tests that the anomalous results were due to the particular preparation of virus agglutinin used in the original tests.

(*b*) *The fowl red cell.*

The influence of the red cell employed in the inhibition test was known to be considerable in the case of the Lee strain of influenza virus B (Stuart-Harris, 1943). No significant differences were encountered by one of us (S.-H.), however, in 1943, using the PR8 virus but Burnet has shown (Burnet and Stone, 1946*a*) that the "sensitivity" of the red cell varies in individual fowls, and that such sensitivity is particularly important in the performance of agglutination tests with vaccinia and ectromelia viruses. A communication was received from Prof. Burnet which suggested that the anomalous results encountered by us could be explained by the fact that we were using insensitive cells, and Anderson, Burnet and Stone (1946) mention that newly-isolated influenza virus A strains are affected by the sensitivity of the actual fowl cells employed in the inhibition test. Tests were therefore made by us with cells from a small number of different fowls. Variations which were encountered in tests in which the same antigen and the same serum were employed together with more than one preparation of fowl cells, were in excess of the two-fold variation inherent in the technique of the test. Thus the titres of 30 sera with the same preparation of either Baumann or Kunz virus were estimated on more than one occasion employing cells from altogether four different fowls. The same titre was obtained five times, a two-fold variation 28 times, and a four-fold variation 11 times. Similar tests with PR8 virus on 27 sera using cells from three different fowls gave the same titre in 17 instances, a two-fold variation in 9 instances, and a four-fold variation once only. It is possible that greater variations still would have been encountered in the case of the newly-isolated viruses if cells had been available from a larger number of fowls. Nevertheless, the tests demonstrated clearly enough that the actual rise in antibody titre to the Kunz virus observed in the inhibition test did depend to some extent on the fowl cell employed, as is obvious from Table III.

TABLE III.—*Comparison of Rises of Antibody with Pairs of Sera using Different Fowl Red Cells.*

Source of sera.	Kunz virus (insensitive R.B.C.'s).	Kunz virus (sensitive R.B.C.'s).	PR8 virus (insensitive R.B.C.'s).
Kunz	2	4	4
Baumann	2	2	8
Woiteki	2	2	2
Kuhlmann	1	4	2
Hesse	2	16	8
Scharnitsky	2	4	8
Mauch	2	½	1
Maurer	4	8	8
Giese	2	4	4
Stoeffler	4	4	16

Figures indicate the ratio of inhibitory titre of convalescent to acute serum in each patient.

This demonstration of a bigger difference between the acute and convalescent serum from an individual patient with one preparation of fowl cells than with another was usually due to obtaining a proportionally lower titre with the acute serum than with the convalescent serum in the presence of the more sensitive red cells. As Anderson, Burnet and Stone (1946) point out, the influence of the sensitivity of the red cell on the inhibition titre is greater in the case of a "normal" serum containing the non-specific inhibitory substance than in the test of a potent immune serum. However, the employment of more sensitive red cells in our tests tended to emphasize the contrast between the titres obtained with PR8 virus and newly-isolated viruses on the convalescent samples of human sera. The fact that with such human sera the titre of inhibition was usually higher with PR8 virus than with a 1947 strain afforded a striking contrast to the results with convalescent ferret sera, and suggested the operation of some other factor than that of the red cell.

(c) *The serum antibody.*

A wider series of tests was first made in which the titres of pairs of human sera from cases of influenza were measured both with the Kunz and PR8 viruses. The results confirmed those mentioned in Table II, and indicated that the greater rise of antibody obtained with the PR8 than with Kunz virus was due not only to a lower titre of inhibition of the acute serum samples with PR8 virus, but also to the fact that the convalescent sera often inhibited this virus more effectively than the Kunz strain (Table IV). It seemed advisable to carry out some neutralization tests in mice with a mouse-adapted virus in order to establish whether or not the inhibition titres of these sera were at variance with the titres of neutralizing antibody. The Kunz virus was therefore adapted to mice by intranasal inoculation of chick lung emulsion from the 7th generation of eggs. No difficulty was encountered in adaptation; lung lesions were obtained which increased in extent on passage, and mice of the 3rd and subsequent passages exhibited fatal infection. In the first neutralization test a series of immune ferret sera and the hyperimmune PR8 horse serum were tested by mixing dilutions of each serum with approximately 500 50 per cent lethal doses of virus, and inoculating the mixtures

TABLE IV.—*Comparison of Kunz and PR8 Antigen in the Examination of Sera showing Rises of Antibody between Acute and Convalescent Samples.*

Numbers of sera (pairs).	Rises of antibody.		
	Same with both viruses.	Greater with PR8 than with Kunz.	Smaller with PR8 than with Kunz.
21	11	9	1

		Ratio of PR8/Kunz titre in various sera.	
		Ratio = 2 or more.	Ratio = 1 or less.
"Acute" specimens	21	8	13
"Convalescent" specimens	21	13	8

intranasally under ether anaesthesia into groups of four mice. The mice were observed for 10 days, and the 50 per cent score end-point calculated on the lung lesions found at autopsy according to the method of Reed and Muench (1938) as suggested by Horsfall (1939). This test (Table V) showed that the Kunz virus, though not identical, was definitely related antigenically to the PR8 virus, but not to either the swine influenza virus or influenza virus B. The failure of the inhibition test to correlate with the neutralization test in the case of the PR8 and Shope ferret sera was noteworthy.

TABLE V.—*Comparison of Inhibition Tests and Mouse Neutralization Tests with Kunz Virus.*

Serum.		Salk inhibition titre.	Mouse neutralization test; 50 per cent. score end-point.	Rise in titre.
PR8 ferret serum		320	50	
PR8 horse serum		20	2·6	
Shope ferret serum		320	<10	
Lee ,, ,,		<20	<2	
Kunz ,, ,,		2560	327	
Kunz	1	80	10	
,,	2	160	>50	2-fold; >5-fold
Baumann	1	80	10	
,,	2	240	>50	3-fold; >5-fold
Kuhlmann	1	20	< 2	
,,	2	80	>50	4-fold; >25-fold
Gillner	1	80	7·6	
,,	2	240	>50	3-fold; >6·5-fold

Figures express reciprocal of the serum dilution. Mouse neutralization test with approximately 500 50 per cent lethal doses of virus. Salk test with 4 minimal agglutinating doses.

A second test was performed in which pairs of human sera were tested against the Kunz virus. A rather weaker preparation of mouse lung emulsion was employed in order to obtain end-points on the "acute" samples of serum. Unfortunately end-points were not obtained with the convalescent samples, which all partially neutralized the virus at the highest dilution of serum which was tested (1 : 50). The results suggested a poor correlation between inhibition

and neutralization titres. In particular, the convalescent serum of Kuhlmann, which never gave an *in vitro* inhibitory titre higher than 1 : 80, was as potent in neutralizing antibody as that of Gillner ,which gave an inhibition at a titre of 1 : 240. No indication that the convalescent human sera were unusually poor in neutralizing antibody to the homologous virus was given by these tests, but a much larger series of neutralization tests would have had to be made in order to establish or refute such a conclusion, and these it was not possible to perform. There seemed, however, reason to doubt that the values obtained in the inhibition test with the Kunz virus were in fact related except in the most general way to the actual antibody content of the serum.

Finally, the results obtained with a number of sera from cases of pneumonia occurring at the time of the outbreaks of influenza but in British civilians must be mentioned. These sera gave high titres of inhibition (640 to 5120) when tested against PR8 virus, and similarly high titres of inhibition against the Kunz virus. Virus was not recovered from the throat or sputum from these cases, in whom the acute sample of serum often had as good a titre of inhibiting antibody as the convalescent sample. Proof was not therefore obtained that these high titres of inhibition in the sera from these cases of pneumonia were due to recent infection with influenza virus, but the results obtained showed that some at least of the human sera collected in the course of the investigation were able to inhibit in high dilution the red-cell agglutination produced by the newly-isolated virus. The generally low titres of inhibition in the convalescent sera from actual cases of influenza contrasted with these results.

DISCUSSION.

The work described above was the outcome of a routine investigation of an outbreak of influenza. It is generally agreed that both serological tests and direct study of the virus agent recovered from the throats of actual patients are desirable in any comprehensive investigation of epidemics of influenza. For if only serological tests are employed, whereas an increase in antibody to standard strains of the influenza viruses will be indicative of an infection with these viruses, absence of antibody increase does not rule out the possibility of a similar infection. Thus the human serological response to infection with an influenza virus may at times be relatively specific, and directed chiefly against the antigen of the particular virus strain concerned in the infection (Magill and Sugg, 1944). Such a state of affairs is probably more frequent in children than in adults (Burnet and Stone, 1946*b*). In such cases serological tests such as the agglutination-inhibition test, in which a single laboratory strain of virus A or B is employed, may fail to reveal the existence of an antibody response to the infecting strain of virus. An example of such a result was recorded by Dudgeon *et al.*, (1946) in the case of sera from cases of influenza B in 1946 at a school in Dorset. Some of the sera exhibited no rise of antibody to the Lee strain of virus B, but antibody responses were detected when the CRA virus recovered during the epidemic was substituted in the Hirst test. The experience recorded in the present work was in complete contrast to this phenomenon. For the sera from many of the 1947 cases of influenza gave a better antibody rise when tested with the PR8 laboratory strain of virus than when tested with viruses recovered from the actual outbreak. Thus instead of an antibody response of a specific character,

the antibody appearing in convalescence seemed to be related to a heterologous antigen (PR8) rather than to the antigen of the infecting virus. Moreover, it appeared impossible to reconcile these results with the fact that ferrets, unlike the human cases, responded to infection with the 1947 virus by the production of an antibody directed more specifically against the homologous virus strain than against PR8 virus.

A full explanation of the present findings has not been obtained by us, but the work has revealed the extraordinary variability of the agglutination-inhibition test, and the lack of correlation in individual sera between the titre of inhibition and the content of neutralizing antibody. There is clearly need for careful scrutiny of the antigen-R.B.C. combination used in the particular test before any conclusion can be drawn in regard to observed differences in inhibition titres of actual sera. Newly-isolated viruses appear to be particularly unsatisfactory in this connection. Francis *et al.* (1946) and also Dudgeon *et al.* (1946) noted that such strains of influenza virus B were very susceptible to inhibition both by heterologous and normal sera. Our own results with these newly-isolated A strains suggested that the strains were less readily inhibited than well-passaged laboratory strains, yet occasional instances of potent inhibition of the new strain by human serum were encountered, and ferret serum devoid of neutralizing antibody was able to produce a considerable degree of non-specific inhibition of agglutination. Only further work with strains of influenza virus recovered from future outbreaks, and possibly a comparison of viruses recovered by more than one method of adaptation, will reveal whether the data recorded represent a relatively-isolated phenomenon or one of general interest.

SUMMARY.

1. Newly-isolated strains of influenza virus obtained from outbreaks of influenza in January, 1947, exhibited a variation in red cell agglutinating activity of the O-D type.

2. Difficulties were encountered in the interpretation of agglutination-inhibition tests with immune animal sera and pairs of sera from cases of human influenza. The virus strains were apparently remotely related to the PR8 standard laboratory strain of influenza virus A. Many of the human patients appeared to have responded to the infection with a better titre of antibodies to the PR8 virus than to the strain responsible for the disease.

3. Variations were encountered in the sensitivity of red cells from different fowls in the agglutination-inhibition test with the newly-isolated viruses.

4. Mouse-neutralization tests with one of the strains suggested that the virus was definitely related to the PR8 virus, and that the neutralizing antibody content of the human convalescent sera was not closely related to the inhibitory action of the serum on the virus *in vitro*.

We wish to thank the Director-General of the Army Medical Service for facilities for the investigation of outbreaks in Army camps, and individual British and German Army officers for their courtesy.

The ferret and horse sera used in the above work were kindly prepared and sent us by Dr. J. A. Dudgeon and Mr. R. E. Glover, of the National Institute for Medical Research.

The streptomycin was a gift of the National Research Council of the U.S.A., and was sent through the courtesy of Dr. J. E. Salk and Dr. Chester S. Keefer.

The expenses of the investigation were defrayed by a grant to one of us (C.H.S-H.) from the Medical Research Council.

Technical assistance was given by Mr. C. H. Bennett.

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SOME PROPERTIES OF POTATO VIRUS X IN LEAF EXTRACTS MADE IN DIFFERENT WAYS.

F. C. BAWDEN AND E. M. CROOK.

From the Rothamsted Experimental Station, Harpenden, Herts.

Received for publication October 11, 1947.

WORKING with tomato bushy stunt, tobacco necrosis and tobacco mosaic viruses, Bawden and Pirie (1944, 1945*a*, 1945*b*, 1946) found that macerating infected leaves and expressing the sap through cloth, the method generally used for extracting plant viruses, left much of the virus contained by the leaves still attached to the fibrous residues. Washing the fibre repeatedly with water did not extract this residual virus, but it could be released by special treatments. Fine grinding in a triple-roller mill released from the fibre about as much of all three viruses as was obtained in the sap, but there was reason to believe that this treatment was also causing some losses. For example, with tomato bushy stunt and tobacco mosaic viruses, more virus was released if the fibre was incubated with trypsin before grinding than if the two processes were made in the reverse order. Also, with tobacco mosaic virus, about twice as much virus was obtained by incubating the fibre with the enzyme mixture obtained from the

alimentary tract of the snail, *Helix aspersa*, as by grinding in the mill. With tobacco mosaic virus, but not with the others, there were also wide differences in the general behaviour of the virus obtained in the extracts made by different methods. No type of extraction gave homogeneous preparations of tobacco mosaic virus particles, and most of the differences could be attributed to the varying proportions of particles of different sizes contained in the different extracts. It was also found that many different treatments could cause smaller particles to aggregate linearly and produce striking changes in the serological and physical properties of the preparations.

The work described in this present paper on potato virus X was undertaken to see whether these results with tobacco mosaic virus were peculiar to it or might also apply to other anisometric viruses. Virus X is less stable than tobacco mosaic virus and is much more difficult to handle in the laboratory or to use in infectivity tests. For these reasons, many of the experiments made with tobacco mosaic virus could not be satisfactorily duplicated with virus X. Nevertheless, the results show that the two viruses do behave similarly in many respects, though there are some interesting differences.

MATERIALS AND METHODS.

Most of the work has been done using tomato (*Lycopersicum esculentum*, var. Kondine Red) and tobacco (*Nicotiana tabacum*, var. White Burley) as host plants for the two virus strains X^K and X^Y. In tomato, X^K produces a severe mosaic with much necrotic spotting, and in tobacco it causes a typical ringspot; X^Y causes a bright yellow inter-veinal mosaic with little or no necrosis in both hosts. Some experiments have also been made with these strains in *Nicotiana glutinosa* and potato (*Solanum tuberosum*, vars. Majestic and Doon Star), and with the virus strain X^S, which causes symptoms resembling those described for X^K, in tomato and tobacco. The different combinations of virus strains and species of host plants did not differ in the manner in which virus could be extracted. The only consistent differences noticed were in the quantities of virus that could be extracted, which depended on both the virus strain and the host. In all the hosts examined, X^K and X^Y occurred at about twice the concentration of X^S. With any one strain, infected tomato plants contained about twice as much virus as tobacco and potato, and *N. glutinosa* was intermediate. The symptoms of infected plants and their virus contents varied with the season, symptoms being most severe and virus content highest in winter and lowest in summer. Regardless of the total virus content reached, however, the proportion of the total that could be extracted by different treatments remained reasonably constant. In other words, plants that gave sap with high virus concentrations also contained more virus in the fibrous residues than did those that gave sap with low virus concentrations.

Tomato plants were inoculated when about 6 inches high and tobacco plants in the 4 to 5 leaf stage, and leaves were picked about a month later when symptoms were well developed. The petioles and main veins were cut out, and the leaf laminae minced by passage through a domestic meat mincer. The sap was then extracted by squeezing through madapollam, when the residual fibre was washed and neutralized as described by Bawden and Pirie (1944). The virus

content in successive washes falls rapidly and three washes using 10 ml. of water per 1 g. of fibre are sufficient to reduce the content below that required to give a visible precipitate with virus antiserum. From 100 g. of leaf laminae there are usually between 70 and 80 ml. of sap and between 20 and 30 g. of fibre, which has a water content of about 70 per cent.

The washed and neutralized fibre, from which no significant amounts of virus can be obtained by further washing or by incubation at 37° C. with water, forms the starting material for the further extraction procedures. For these the same triple-roller mill, enzyme preparations, and general techniques were used as those described by Bawden and Pirie (1944, 1946). Some attempts were made to measure the amount of virus extracted by different methods by isolating and weighing purified virus, but these were largely unsuccessful. Whether purification was attempted by methods involving precipitation with salts and acid, by ultra-centrifugation, or a combination of both, there were large losses of virus, mainly because it separated in insoluble states, so that quantitative recovery was impossible. Estimations of virus content in the different extracts were therefore made largely by serological methods, using antisera prepared by the intravenous injection of rabbits with purified virus X. Precipitin tests were made at 50° C., 1 ml. of antiserum at a constant dilution of 1 in 200 being added to a series of tubes each containing 1 ml. of test fluid of various dilutions. The greatest dilution to give a precipitate visible to the naked eye after 3 hours' incubation with the antiserum was taken as the precipitation end-point. This is not altogether a reliable index of virus content, because the same quantity of virus in different physical states can give different precipitation end-points, but here we shall be concerned only with differences in quantity that are beyond this experimental error.

Infectivity tests were made with the strain X^K on tobacco and *N. glutinosa*. In such tests the degree of accuracy that can be obtained is far less than with tobacco mosaic virus, for although on some plants this strain gives discrete, well-defined local lesions, on others the lesions are diffuse and difficult to count accurately. To reduce these variations as far as possible, different extracts to be compared were applied to half-leaves using a Latin square design or some similar random distribution of treatments.

Properties of virus extracted by milling.

The chromoprotein contained in the deep green solutions that are produced when milled fibre is extracted with water, is readily denatured by freezing and thawing, and frozen, centrifuged mill extracts are clear and pale green or yellow. With tobacco mosaic, tomato bushy stunt and tobacco necrosis viruses, the virus content of solutions made by extracting milled fibre with three times its own weight of water, that is, about the equivalent volume of sap, is approximately the same as the virus content of the sap itself. With potato virus X, much more virus is obtained in the sap than in mill extracts made with this ratio of water to fibre; the precipitin titres of such mill extracts are only one-thirtieth or less of those of sap. To get extracts with a sufficient virus content for their serological behaviour to be studied, the milled fibre must be extracted with not more than its own weight of water. Table I shows one comparison, the results of which are typical of many made with the various virus strains and host plants,

between the properties of virus occurring in sap and mill extracts obtained from tomato leaves infected with X^K. The washed fibre was passed twice through the roller mill, and then extracted three times with half its own weight of water. At each extraction the water and fibre were thoroughly mixed and allowed to stand for 30 minutes before separating. The sap and mill extracts were frozen over-night and clarified by centrifuging before testing.

TABLE I.—*Comparison of Virus X obtained in Sap and Mill Extracts.*

Extract.	Time for first appearance of precipitation.	Dilution of antigen optimum.	Precipitation end-point.	Relative virus content.*
Sap	2 minutes	1 : 4	1 : 1024	380
1st extract of milled fibre	30 ,,	1 : 32	1 : 128	8
2nd extract	30 ,,	1 : 16	1 : 64	4
3rd extract	30 ,,	1 : 4	1 : 16	1

* The values given for the relative virus contents are the observed precipitation end-points multiplied by factors to bring them to a standard ratio of 1 ml. of fluid from 1 g. wet weight of washed fibre. Antiserum used at 1 : 200.

Because of qualitative differences between the serological behaviour of mill extracts and sap, the figures given for relative virus contents are probably biased in favour of the sap by a factor of from 2 to 4. Nevertheless, when due allowance is made for such errors, it is obvious that the sap equivalent to 1 g. of washed fibre contains many times as much virus as can be released by milling. The qualitative differences between the two types of extract resemble those previously found with tobacco mosaic virus, the virus in the sap behaving like a flagellar antigen and that in the mill extracts approximating more to the character of a somatic type antigen. When sap is mixed with antiserum, precipitation occurs rapidly over a wide range of antigen/antibody ratios, and the precipitates are diffuse, with a fluffy open structure. In the mill extracts, on the other hand, precipitation occurs more slowly and over a more restricted zone, inhibition of precipitation being particularly pronounced in the region of antigen excess, and the precipitates themselves are more granular and compact. With tobacco mosaic virus these differences in serological behaviour are correlated with size of virus particle, preparations containing predominantly long rods behaving like

CAPTIONS TO MICROGRAMS.

Electron micrograms of potato virus X (strain X^K) showing different states of aggregation resulting from various methods of preparation.

Two typical micrograms of each type of preparation are shown, each pair being chosen to indicate the range of particle sizes normally encountered in such preparations.

Sap and mill extracts were clarified by freezing and centrifuging, but not otherwise prepared. All specimens were made by placing on supporting membranes a drop of solution containing virus at a concentration of between 10^{-5} and 10^{-6} g. per l.

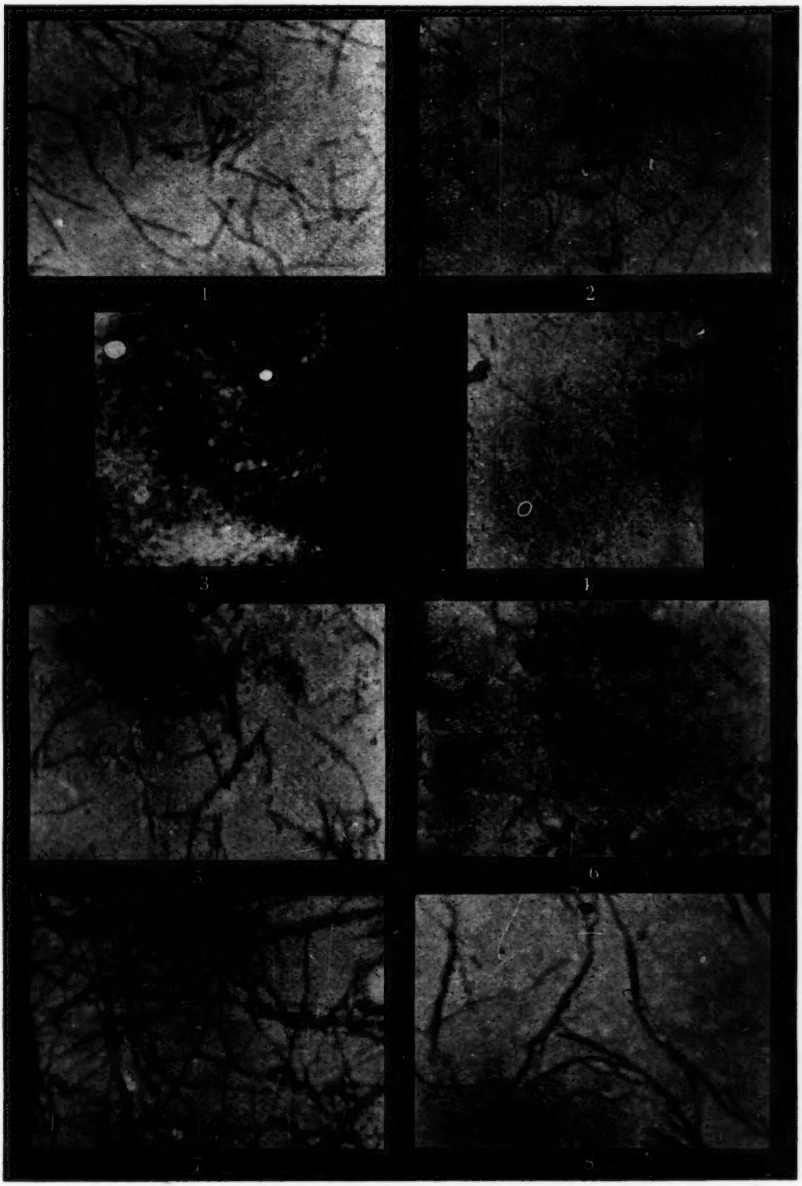
Negatives obtained in R.C.A. Type EMB2 microscope, accelerating potential 45 kV. Final magnification approximately × 20,000—i.e., 1 cm. = 500 mμ.

FIG. 1 and 2.—Particles occurring in clarified sap.

FIG. 3 and 4.—Particles occurring in mill extracts from fibre.

FIG. 5 and 6.—Particles in mill extracts of Fig. 3 and 4 after incubation for 16 hours at 37° C. at pH 7 with normal tobacco sap at a final dilution of 1 in 5.

FIG. 7 and 8.—Particles in purified virus preparations.



Bawden and Crook.

UNIV. OF MICH.

flagellar antigens and those with shorter particles behaving more like somatic antigens (Bawden and Pirie, 1945*b*; Crook and Sheffield, 1946). This also seems to be true with potato virus X, for electron micrograms show that mill extracts contain few rods longer than 250 mμ, whereas in sap most of the particles are longer than this, some being longer than 5,000 mμ. (Fig. 1, 2, 3, 4). In both types of extract, however, there is much variation in the length of particles and in neither are the particles stable. The particles of virus X as photographed in the electron microscope give the impression of being more flexible than those of tobacco mosaic virus. This has already been commented upon by Takahashi and Rawlins (1946), who also found some variation in particle length, but concluded that the most probable length for a number of strains was 500–600 mμ. Our micrograms, however, show that the range of particle lengths is as great as with tobacco mosaic virus (Crook and Sheffield, 1946), and with neither virus does the electron microscope give any evidence about the size of the smallest particle capable of causing infection.

As with tobacco mosaic virus, the small particles in virus X preparations can aggregate linearly, and, as they increase in length, there are corresponding changes in the behaviour of the preparations. In sap, aggregation is most conveniently studied by following the increase in intensity of anisotropy of flow, for the length of the particles is already such that the serological reactions are but little affected by any increases in length. The amount of anisotropy of flow shown by freshly-prepared sap, after clarification by freezing and centrifuging, varies with the host plant and the virus strain used. With all, however, it can be greatly increased by such treatments as heating to 60° C. or merely allowing to age, treatments that are also effective in causing aggregation with tobacco mosaic virus.

In mill extracts the virus content is insufficient to give any anisotropy of flow, but treatments that cause aggregation can conveniently be studied by following changes in behaviour during precipitin tests. Electron micrograms show that the changes from somatic to flagellar type behaviour with these extracts also are accompanied by increases in the average length of the virus particles (Fig. 5, 6). Bawden and Pirie (1945*b*), working with preparations of small particles of tobacco mosaic virus separated by differential ultra-centrifugation, found that many different treatments caused aggregation. Working with unfractionated mill extracts of potato virus X, we have found that whereas a few of these cause aggregation, many do not. Whether this is because of a difference between the viruses, or because the preparations of virus X used are both more dilute and contain a greater ratio of other materials to virus than the partially purified preparations of tobacco mosaic virus, there is no information.

The relative instability of virus X is a complication in attempting to compare the efficacy of different treatments in causing aggregation with virus X and tobacco mosaic virus. Some treatments that aggregate tobacco mosaic virus destroy virus X. For example, incubation with trypsin is the most effective method of aggregating tobacco mosaic virus, but it hydrolyses virus X. With this treatment, however, aggregation proceeds more rapidly than hydrolysis and, provided the time of incubation is not extended, aggregation can be demonstrated. When mill extracts are incubated with 0·8 per cent B.D.H. trypsin solutions at pH 7 for 90 minutes, their serological behaviour is altered so that they give flagellar-

type floccules, precipitate rapidly with antiserum and there is no pronounced zone of inhibition in the region of antigen excess. During this time about one-half the virus is hydrolysed, so that there is the unusual phenomenon of a fall in antigen concentration being accompanied by an increased rate of precipitation. Incubation in these conditions for a longer period leads to the destruction of the virus and failure to precipitate with antiserum. Thus, it seems that, as a preliminary to hydrolysis, the action of trypsin first leads to an increase in the size of particles. Treatments such as incubation with phosphate buffer and chloroform, which aggregate tobacco mosaic virus, but do so less readily than trypsin, gave inconclusive results with virus X, probably because such treatments denatured the virus more rapidly than they caused any unequivocal changes in the serological behaviour. In some experiments in which mill extracts were incubated with phosphate buffer alone, there was evidence of some aggregation, but, in the absence of an antiseptic, this could have resulted from the activities of contaminating bacteria.

The most suitable method that we found of demonstrating aggregation in mill extracts of virus X is incubation at pH 7 and 37° C. with clarified sap from healthy plants (Fig. 5, 6). Sap from tobacco, tomato and *N. glutinosa* is all equally effective; incubation of mill extracts containing a third of a volume of healthy sap, clarified by freezing, thawing and centrifuging, for 16 hours has always changed their serological behaviour to that characteristic of aggregated virus. Analogous changes also occur when mill extracts are allowed to age, but the rate at which aggregation occurs varies greatly from preparation to preparation; the presence of an antiseptic does not prevent this aggregation, but it does reduce the rate at which it occurs.

One treatment that is effective in causing aggregation of the virus in mill extracts of tobacco mosaic virus is heating to 60° C. at pH values between 5 and 6. This has consistently failed with mill extracts of virus X. In testing treatments that involve lowering the pH value, however, it is essential to neutralize before making precipitin tests as the acid itself causes aggregation and affects the character of the precipitin reaction. In clarified sap the aggregation caused by acidification to pH 5·5 or lower can usually be detected by an increase in the amount of anisotropy of flow shown when the fluids are shaken in polarized light, or by the development of a satin-like sheen when shaken and observed by reflected light. In both sap and mill extracts the aggregation caused by short exposures to pH values between 4 and 5·5 is fully reversible, and neutralization restores the original optical and serological properties of the extracts. The effect of some treatments on the serological properties of mill extracts are given in Table II.

Liberation of virus X by snail enzymes.

The ratio of the amount of virus X obtained in mill extracts to the amount present in sap is much smaller than the ratio found with tobacco mosaic, tomato bushy stunt or tobacco necrosis viruses. This could mean either that virus X is distributed in infected tissues in a different manner from the others, so that much more of the total virus content of the leaves is released into the sap, or that the distribution is similar, but milling is less effective in releasing virus X. The results of incubating washed fibre with snail enzymes show that the second explanation is the more probable, for this treatment liberates many times more

TABLE II.—*The Effect of Various Treatments on the Serological Behaviour of Mill Extracts of Virus X.*

Treatment of mill extract.	Time for first appearance of precipitation.	Dilution of extract to precipitate most rapidly.
Control, incubated alone . .	30 minutes .	1 : 32
Incubated 90 min. with 0·8 per cent trypsin	2 ,, .	1 : 1
Incubated 16 hours with healthy sap	2 ,, .	1 : 1
Heated to 60° C. at pH 5·8 .	30 ,, .	1 : 32
Incubated with snail enzyme .	30 ,, .	1 : 32
Unincubated, tested at pH 5 .	4 ,, .	1 : 2
Unincubated, tested at pH 5·5 .	12 ,, .	1 : 8

Unless otherwise stated all serological tests and incubations were made at pH 7; antiserum used at 1 : 200.

virus than does milling. The virus liberated also has different properties, suggesting that the average length of the particles is greater than in freshly-expressed sap. The virus extracted by incubation with the snail enzymes shows anisotropy of flow at greater dilutions than that in sap, and in its serological behaviour resembles that of highly aggregated, purified virus preparations (Fig. 7, 8).

Incubations were made at 37° C. for 48 hours. Samples of 1 g. of washed fibre were mixed with 0·5 ml. of pH 7 Michaelis' veronal buffer, 1 ml. of the snail enzyme preparation and 1 ml. distilled water, with chloroform added as an antiseptic. During incubation the mixture becomes acid and repeated additions of NaOH are needed to keep the pH at 7. After incubation, 4 ml. of water were added and the mixtures were left to soak for 30 minutes before being clarified by 20 minutes' centrifugation at 3000 r.p.m. As with tobacco mosaic virus, not all the virus liberated during the incubation dissolves in the first extraction, and a second wash of the fibre with 5 ml. of water may contain almost as much virus as the first. Similarly, more than one incubation with snail enzyme preparation is necessary to release all the virus that can be obtained. A second incubation with a fresh lot of enzyme always gives a yield almost as good as the first, but the amount obtained by a third incubation is relatively small. Whether this means that all the virus originally present has now been released, or that incubation in these conditions for longer than 4 days destroys virus X, is unknown. Comparisons made on the basis of precipitation end-points of snail extracts from washed fibre suggest that the virus content of the washed fibre is at least equal to the virus content of the sap derived from that fibre. Two such comparisons are given in Table III. In these tests, the successive extracts and washes of incubated fibre were pooled, and the mixed extracts and sap titrated against antiserum. The relative virus contents were then calculated by multiplying by appropriate factors to bring them to a standard reference of virus from 1 g. of washed fibre. The actual virus contents of the two saps were approximately 0·15 mg. per ml. for X^S in tobacco and 1 mg. per ml. for X^K in tomato. Thus a kilogram of tomato leaf laminae, with a dry weight of 100 g., contained at least 0·3 g. of X^S and 2 g. of X^K.

TABLE III.—*Comparison of Virus X Extracted by Different Methods.*

Strain and host.	Extract.	Relative virus content.*	Infectivity. Average number of lesions per leaf at	
			10^{-5}.	10^{-6}.
X^S in tobacco	Sap	96	100	19
	Mill	3	83	27
	Snail	128	97	20
X^K in tomato	Sap	600	66	15
	Mill	20	50	12
	Snail	500	70	15

* Relative virus contents calculated by multiplying the observed precipitation end-points by factors to bring all of them to a standard ratio of 1 ml. of extract from 1 g. wet weight of washed fibre.

The snail enzyme preparation is a powerful inhibitor of infectivity, so that comparative infectivity tests with sap, mill extracts and snail extracts cannot be made at all reliably. Some tests, however, have been made with virus purified from the different kinds of extracts, and two of these are shown in Table III. With tobacco mosaic virus, Bawden and Pirie (1946) found that the virus remaining in the fibrous residues was less infective than that contained in the sap. We have found no such differences with virus X. The purified preparations that have been made from different kinds of leaf extracts have not varied consistently in their infectivity, elementary analyses, physical properties or precipitation end-points.

After milling, no further virus can be liberated from the fibre by incubation with snail enzymes. The virus liberated by the two treatments, therefore, is not complementary and milling makes much of the virus that is normally extractable by snail enzymes irrecoverable. With tobacco mosaic and tomato bushy stunt viruses, milling also renders some virus irrecoverable, but with these the loss is of the order of 50 per cent of the virus content of the fibre whereas with virus X it is more than 90 per cent. Bawden and Pirie (1946) showed that these two other viruses could become attached to some constituent of the fibre during the process of milling, but they suggested that, unless there was attachment to some substrate unaffected by trypsin and snail enzymes, the total loss could only be explained by the fact that milling was also destroying some virus. The results with virus X strengthen this suggestion, for virus X is less stable than the other viruses. Particularly it is more sensitive to heat, and so might be expected to be more easily denatured by friction between the rollers, but there is no reason to assume that it would be more likely to combine with other materials during milling. With the other viruses, a preliminary incubation of the washed fibre with trypsin was beneficial in increasing the amount of virus obtained by milling, presumably because the enzyme removes something from the fibre that is responsible for the loss of virus. This treatment is not applicable to virus X. After incubation with trypsin, no virus X can be obtained either by milling or by subsequent incubation with snail enzymes, presumably because the virus in the fibre is hydrolysed by the trypsin. If milling also is an inactivating treat-

ment, its effects resemble those of heating (Bawden 1941), and not those of treatments that cause loss of infectivity without affecting serological activity. The small amount of virus obtained in the mill extracts seems to be, weight for weight, as infective as that occurring in leaf extracts made by the other methods, indicating that any particles that may have been affected are disrupted and lose simultaneously both infectivity and serological activity.

Inactivation of virus X by pH 7 phosphate buffer.

To gain some evidence as to whether the virus obtained in snail extracts was a measure of the total extractable virus in leaf fibre, or whether incubation was likely to be causing losses, purified virus preparations were incubated with the various substances with which the fibre was usually incubated. It was then found that incubation with phosphate alone led to a fall in the serological titre, and that when chloroform was also present a white gelatinous precipitate separated; in the presence of snail enzyme, however, neither phenomenon was noticed.

As inactivation of virus X by phosphate buffers has not previously been recorded, the phenomenon was investigated further. Table IV gives the results of one representative experiment in which a purified preparation of X^K was incubated in various conditions. Incubation with water alone or with chloroform has very little, if any, effect on the precipitation end-point. Phosphate alone causes inactivation, but the effect is much increased by the simultaneous presence of chloroform. The reaction at 37° C. appears to occur at a fairly uniform velocity, but almost ceases after 16 hours, there being a small equilibrium residuum of serologically active virus. The ratio of residual to initial virus is approximately constant after 16 hours independently of the initial concentration, suggesting that the initial velocity of inactivation is proportional to the virus concentration. The degree of inactivation is affected by the concentration of phosphate and chloroform. Much the most effective conditions for denaturation are to have liquid chloroform present, i.e., to have the fluid saturated and a layer of undissolved chloroform in the bottom of the vessel. The rate of inactivation depends greatly on temperature. Little if any change occurs at room temperature or below, although there is often slight clouding of the virus solution when phosphate is added. Inactivation at 37° C. is such that more than 90 per cent of the initial virus is destroyed in 16 hours, whereas at 50° C. denaturation is substantially complete in two hours. The pH of the reaction mixture is also important. Thus, under conditions which result in 96 per cent inactivation of the virus at pH 7, no change is detectable at pH 5·5.

During incubation with phosphate, loss of infectivity occurs more rapidly than denaturation and loss of serological activity. After 8 hours' incubation of one preparation of X^K at pH 7 with 0·1 M phosphate buffer at 37° C., for example, the infectivity had fallen to about a hundredth of the original, although the precipitin titre had fallen only to one-eighth; and after 16 hours' incubation the preparation gave no local lesions on tobacco, although it still retained about 2 per cent of its original serological activity.

In the presence of chloroform a precipitate separates during incubation if the initial concentration of virus is higher than 0·1 per cent. Under these conditions the nucleic acid content of the precipitate appears to be substantially reduced. For example, from one specimen of virus containing 0·3 per cent phosphorus

and 8·1 per cent carbohydrate, a precipitate was obtained containing only 3·7 per cent carbohydrate and less than 0·1 per cent phosphorus. That is, both had been reduced to approximately one-third of the initial.

TABLE IV.—*The Inactivation of Virus X by Incubation with Phosphate and Chloroform.*

A. *Separate and Combined Effects of 0·1 M Phosphate and Chloroform.*

Virus incubated with—	Percentage inactivation.
Water	0
Saturated chloroform	20
Liquid chloroform layer	36
Phosphate alone	68
Phosphate + saturated chloroform	68
Phosphate + liquid chloroform layer	98

B. *Effect of Time of Incubation.*

Time.	Percentage inactivation.
0 hours	0
2 ,,	36
4 ,,	76
8 ,,	84
16 ,,	96
24 ,,	96

C. *Effect of Initial Virus Concentration.*

Virus concentration.	Percentage inactivation.
0·15 g./l.	96
0·30 ,,	96
0·60 ,,	96

D. *Effect of Phosphate Concentration in Saturated Chloroform.*

PO_4 concentration.	Percentage inactivation.
0	0
0·004 M	36
0·02 M	50
0·1 M	68

E. *Effect of Various Substances in Preventing Inactivation by phosphate.*

*Substance.	pH.	Percentage inactivation.
Control	7	97
Control	5·5	0
Snail enzyme	7	0
Tobacco protease	7	68
Tobacco protease	5·5	100
Normal leaf sap	7	0
Albumin	7	88
Gelatin	7	50
Casein	7	68

F. *Inactivation by 0·1 M Solutions of Various Salts.*

Salt.	Percentage inactivation.
Nil	0
Orthophosphate	96
Pyrophosphate	94
Veronal (Michaelis)	50
Citrate	94
Tartrate	92
Borate	50
Oxalate	0
Salicylate	50

* Final concentrations of added substances per 1·8 ml. of fluid : 0·3 ml. of snail enzyme preparation ; 0·2 ml. of sap from healthy tomato leaves ; 1,500 units (Tracey, 1947) of tobacco leaf protease ; 0·5 per cent of albumin, gelatin and casein.

All incubations were at 37° C. ; except in series B, time of incubation was 16 hours ; except in series E, pH of fluid was 7 ; except in series C, initial virus concentration was 0·5 mg. in 1·8 ml. ; except in series D and F, phosphate concentration was 0·1 M ; except in series A and D, a layer of liquid chloroform was present. Inactivation is calculated from the percentage of the initial virus remaining active as estimated from the precipitin titres of the incubated fluids.

As snail enzyme preparations were found to protect virus X against inactivation by phosphate, other substances were tested for their protective action. Most of these gave protection in greater or less degree, the most effective being proteins or materials like plant sap which are rich in protein. One result of interest was obtained with a concentrated preparation of tobacco leaf protease of the papain type (Tracey, 1947). At pH 7 the presence of this enzyme preparation protected the virus against the inactivating action of phosphate, whereas, at pH 5·5, where phosphate does not inactivate, the protease itself destroyed the virus. All purified preparations of virus X that have been tested were partially inactivated when incubated for 16 hours at 37° C. with 0·1 M phosphate buffer at pH 7 in the presence of chloroform, but the amount of inactivation has varied somewhat with different preparations. Consistent results are obtained in repeated tests with the same preparation, and the variations with different preparations presumably arise because they contain different impurities, or different amounts of one impurity, that act as stabilizers.

Incubation of clarified sap with added phosphate at pH 7 and 37° C. leads to no fall in precipitin titre, but mill extracts are as susceptible as the most highly purified virus preparations. This shows clearly that the mill extracts contain no appreciable amounts of protective substances, which is in agreement with their low solid content; their dry weight rarely exceeds 0·3 g./l., of which 80 per cent is precipitable with trichloracetic acid.

Inactivating effects are not confined to phosphate buffers. Of the buffers and salts tested, only veronal and borate buffers were relatively harmless. As borate is not a satisfactory buffer at pH 7, veronal was selected for use when incubating fibre with snail enzymes. The protective effect of snail enzymes, however, is so great that it is unlikely that there is any significant loss of virus during incubation.

DISCUSSION.

The main points that call for comment are the state in which virus particles occur in the plant and the relationship between the virus occurring in sap and that remaining in the fibrous residues, about which there is little unequivocal evidence. Virus X occurs predominantly in the form of small particles only in mill extracts, and as there is good reason to believe that milling causes destruction of the virus, these small particles could be regarded as partial break-down products. On the other hand, the infectivity of mill extracts suggests that, if these small particles are artefacts and not representative of the condition in which they normally occur in the plant, the disruption has not gone far enough to destroy infectivity. Also, any disruption seems to be reversible, for the particles in mill extracts can be made to aggregate so that their general properties become indistinguishable from those of particles in sap and in snail extracts.

Incubating the fibrous leaf residues with snail enzymes releases virus in the form of greatly elongated particles. The snail enzymes seem to have no aggragating power when incubated with mill extracts, and it might seem reasonable at first sight to assume that the condition of virus particles in snail extracts would be similar to that in which they occur in infected cells. However, this is far from unequivocal evidence. The effects of healthy sap in causing aggregation in mill extracts leave no doubt that plant tissues contain substances that can

cause aggregation, and during incubation of the fibre with snail enzymes there could be ample opportunity for these to be active. If the condition of the virus in snail extracts of fibre is typical of the particles as they occur and are produced in the cells, it is also necessary to assume that the mincing process needed to release sap is also causing disruption of larger particles, for the average length of the particles in snail extracts is greater than that of the particles in freshly-expressed sap. The particles in the sap, as with tobacco mosaic virus, are of variable lengths, but we have made no attempt at fractionation to determine whether preparations of virus X particles of different sizes also differ in their infectivities. In the conditions obtaining in sap, all the particles readily increase in length and it seems reasonable to assume that they are all produced by the joining together of similar, though possibly not identical, units. Thus, although there is no doubt that virus X can exist in particles of greatly differing lengths, there is no positive evidence to indicate the size of any basic infective particle from which the larger ones might be produced by aggregation; nor is there anything conclusive to indicate the size and condition of the virus as it is produced and exists in infected cells.

With tobacco mosaic virus, Bawden and Pirie (1945*b*, 1946) found that the virus from the fibre was, weight for weight, less infective than that in the sap. This led them to suggest that the two lots of virus were qualitatively different and that the virus in the fibre might be combined with some host constituent which rendered it insoluble. The release of the virus by milling and snail enzymes would, on this hypothesis, be explicable because the treatments destroyed the constituent with which the virus was combined. While our results do not refute this suggestion, they can equally readily be interpreted on the basis that the virus obtained from the fibre is similar to that in the sap, but merely comes from different cells. During the ordinary mincing process, all the cells seem to be damaged sufficiently for the water they contain to be released, for if the mincing is repeated or the fibre is ground very finely, there is no significant increase in the amount of sap obtained. On the other hand, mincing may not damage all the cells sufficiently for the virus in them to be able to escape with the sap. If this is so, when the sap is expressed it will contain the virus from the cells that have been sufficiently disrupted to permit its release, but the virus in the others will remain within the cell walls and merely become more concentrated as the water is expressed. Then the effects of milling and incubation with snail enzymes will be simply to disrupt the walls of these relatively undamaged cells sufficiently for the virus to be set free and extracted in added water. There is no positive evidence for one view rather than the other, but over a wide range of different virus contents, with both tobacco mosaic virus and virus X, the ratio of virus in the sap to that retained in the fibre after mincing is reasonably constant; this does perhaps suggest that mincing may be opening a constant proportion of cells rather than that the two lots of virus are produced differently or vary in any other qualitative manner.

SUMMARY.

By incubating fibrous residues from leaves infected with potato virus X with enzymes from the alimentary tract of snails, as much virus is obtained as occurs

in sap. About 4 per cent of the virus extracted by snail enzymes is released by grinding the fibre finely in a triple-roller mill.

The virus in the different kinds of extracts does not differ greatly in infectivity, but the sizes of particles vary widely. The particles in mill extracts are short and give somatic-type precipitin reactions. In snail extracts, the particles are greatly elongated; in freshly-extracted sap, particle length is variable, but the average is greater than in mill extracts; both extracts give typical flagellar type precipitin reactions. The virus particles in sap vary in length and readily aggregate linearly.

Incubating mill extracts with healthy plant sap or trypsin causes the small particles to aggregate and changes their precipitin reaction from somatic to flagellar type.

Virus X in mill extracts or in purified preparations, but not in sap, is inactivated by incubation with pH 7 phosphate buffer and chloroform. This inactivation is prevented by snail enzyme preparations, healthy leaf sap and some other protein-containing solutions.

The virus content of infected plants varies with different hosts and virus strains. The largest was found with X^K in tomatoes, where the virus amounted to 2 per cent of the dry weight of the leaves. There is no unequivocal evidence on either the size of the particles as they exist in the host cells or the relationship between the virus occurring in sap and that remaining in the fibrous residues.

We wish to thank Dr. A. Kleczkowski for making the antisera used, Dr. F. M. L. Sheffield for taking the electron micrograms, and Mr. M. V. Tracey for supplying us with the preparation of tobacco leaf protease.

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INVESTIGATION INTO THE PRODUCTION OF ANTIBACTERIAL SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF THE EIGHTH 100 SPECIES, ALL BASIDIOMYCETES.

W. H. WILKINS.

From the Mycology Laboratory, University Department of Botany, Oxford.

Received for publication October 28, 1947.

THIS paper, which deals with the eighth "100 species" of fungi in the series (for references see Wilkins, 1947), is perhaps better regarded as the fourth 100 of the species of Basidiomycetes, as work has for some time been concentrated in this group, and is rather distinct from work on the previous groups.

The cultures were all obtained from the Centraalbureau voor Schimmelcultures, Baarn, and have, therefore, no symbols indicating their source. They were grown in solid media, and those that reacted positively to an initial "strip" test (Wilkins and Harris, 1944) were grown in liquid media and tested by the "hole" method (Wilkins and Harris, 1943). Both tests were against the representative organisms *Bacterium coli* and *Staphylococcus aureus*.

The positive results of the second test are arranged in two classes: (1) "strongly positive," when the diameter of the total area of inhibition was 20 mm. or more, and (2) "weakly positive," when it was 15–20 mm. The "strongly positive" fungi are recorded in greater detail.

LIST OF FUNGI EXAMINED.

Strongly Positive.

	Result of test on liquid medium.			
			Zone diameter (mm.).	
	Time in days.	Medium.*	*Bact. coli.*	*Staph. aureus.*
Amanita Vittadinii (Moretti) Vitt.	10	P.D.	..	25
Clitocybe aurantiaca (Wulf.) Studer	20	M.	..	23
C. flaccida Sow.	5	P.D.	..	21
C. fragrans Sow.	30	P.D.	25	34
C. Hirneola Fr.	20	M.	..	20
Coniophora Bourdotti Bres.	5	M.	..	25
Coprinus erethismus Heim.	25	M.	17	36
C. Friesii Quél.	25	M.	18	30
C. miser Karst.	25	P.D.	..	29
C. picaceus Bull.	15	M.	17	25
C. plicatilis Curt.	15	P.D.	..	23
C. quadrifidus Pk.	15	M.	17	25
C. sphaerophorus Buller	10	P.D.	19	40
Corticium centrifugum (Lév.) Tul.	30	M. or P.D.	29	25
C. effuscatum Cke. & Ell.	15	M.	..	20
Naucoria semiorbicularis (Bull.) Fr.	20	M.	..	22
Pholiota erebia Fr.	25	M.	..	27
Tricholoma panaeolum Fr.	20	M. or P.D.	..	24

* M. = malt; P.D. = potato dextrose.

Weakly Positive.

Clitocybe cyathiformis Fr.; *C. olearia* D.C.; *Clitopilus abortivus* B. & C.; *Collybia collina* (Scop.) Fr.; *Coniophora arida* Fr.; *C. cerebella* Alb. & Schw.; *C. cystidiophora* Falck; *C. merulioides* Falck; *C. sulfocata* (Pk.) Massee; *Coprinus auricomus* Pat.; *C. comatus* Fr.; *C. fimentarius* (L.) Fr. var. *macrorhizus* Pers.; *C. vermiculifer* Josserand; *Corticium anceps* (Bres. & Syd.) Gregor; *C. bisporum* Schroet.; *C. galactinum* (Fr.) Burt.; *Lepiota naucina* Fr.; *Mycena cephalotricha* Josserand; *Pholiota aegerita* (Brig.) Quél.; *P. marginata* Batsch.

Negative.

Amanita rubescens Fr.; *A. spissa* Fr.; *Armillaria fusipes* Petch; *A. mellea* (Vahl) Quél.; *A. mucida* Schrad.; *Auricularia mesenterica* (Dicks.) Pers.; *Boletus flavidus* Fr.; *B. granulatus* (L.) Fr.; *B. luridus* (Schaeff) Fr.; *Calocera cornea* (Batsch) Fr.; *Claudopus nidulans* (Pers.) Pk.; *Clitocybe gallinacea* Scop.; *C. illudens* Schw.; *C. nebularis* (Batsch) Quél.; *C. rivulosa* (Pers.) Fr.; *C. tabascens* (Scop.) Bres.; *Collybia acervata* Fr.; *C. butyracea* (Bull.) Fr.; *C. conigena* (Pers.) Fr.; *C. dryophila* (Bull.) Fr.; *C. fusipes* (Bull.) Berk.; *C. radicata* (Rehl.) Berk.; *Coprinus atramentarius* (Bull.) Fr.; *C. Boudieri* Quél.; *C. dilectus* Fr.; *C. domesticus* Pers.; *C. ephemerus* (Bull.) Fr.; *C. micaceus* (Bull.) Fr.; *C. narcoticus* (Batsch) Fr.; *C. radians* (Desm.) Fr.; *C. similis* Berk. & Br.; *C. stercorarius* Fr.; *C. sterquilinus* Fr.; *C. urticaecola* (Berk. & Br.) Buller; *Corticium alutaceum* (Schrad.) Bres.; *C. coeruleum* (Schrad.) Fr.; *C. fuciforme* (Berk.) Wakef.; *Entoloma sericeum* (Bull.) Fr.; *Flammula gummosa* (Lasch.) Fr.; *F. lenta* Pers.; *Galera rubiginosa* Pers.; *Hypholoma velutinum* (Pers.) Fr.; *Lepiota gracilenta* (Krombh.) Fr. sensu Ricken; *L. lutea* With.; *L. procera* Scop.; *Marasmius oreades* Fr.; *Mycena aetites* Fr.; *M. galopus* Pers.; *M. inclinata* Fr.; *M. latifolia* Peck; *M. rorida* Fr.; *Naucoria vervacti* Fr.; *Omphalia Mairei* Gilbert; *Panaeolus acuminatus* Fr. sensu Ricken; *Pholiota paludosa* Lange; *Pluteus cinereus* Quél.; *Psalliota haemorrhoidaria* Fr.; *Psathyrella disseminata* (Pers.) Fr.; *Stropharia coronilla* (Bull.) Fr.; *S. semiglobata* Batsch; *Tricholoma nudum* (Bull.) Fr.

The above list is, I think, self-explanatory. Detailed comment on the antibacterial activity of Basidiomycetes in general, within the limits of this investigation, will be postponed until a later date.

I am indebted to the Medical Research Council for a grant enabling me to employ Research Assistants, and in this case I am grateful for the help of Miss G. M. Trevaldwyn, M.A., Miss J. W. Hawkes, B.Sc., and Miss D. E. Walker, B.Sc. The Technical Assistant, Miss J. Rose, has also given valuable assistance.

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LICHENIFORMIN, AN ANTIBIOTIC SUBSTANCE FROM *BACILLUS LICHENIFORMIS*, ACTIVE AGAINST *MYCOBACTERIUM TUBERCULOSIS*.

R. K. CALLOW, R. E. GLOVER, P. D'ARCY HART AND G. M. HILLS.

From the National Institute for Medical Research, Hampstead and Mill Hill, London, and the Microbiological Research Department, Experimental Station, Porton.

Received for publication November 1, 1947.

LICHENIFORMIN is an antibiotic isolated as a hydrochloride from the spore-forming aerobe *Bacillus licheniformis* (Weigmann, *emend.* Gibson). Its preparation and chemical and biological properties have already been outlined in preliminary reports (Callow and Hart, 1946; Hart and Hills, 1947; Callow, Glover and Hart, 1947). This communication gives experimental details and further observations.

METHODS.

Unless otherwise stated, the preparations were from a single stock strain of *B. licheniformis* (identified by Dr. T. Gibson). After trials of a number of culture media and conditions, two procedures were finally followed for small scale and larger scale production, respectively; in both, synthetic media were employed.

Production of Licheniformin.

Small scale production.

Maintenance.—Cultures were grown in capped test-tubes for 48 hours at 37° C. on the medium given below, and could then be maintained for several months at room temperature: (1) K_2HPO_4, 1 g.; (2) sodium citrate, 0·5 g.; (3) $MgSO_4$, $7H_2O$, 1 g.; (4) asparagine, 5 g.; (5) iron ammonium citrate (scales), 1 ml. 1·7 per cent solution; (6) glycerol, 70 ml.; (7) agar, 20 g.; (8) distilled water to 1 l.; pH (after autoclaving), 7·0–7·2.

Inoculation.—A 5/8 in. diameter test-tube of 5 ml. Hartley pancreatic digest broth was inoculated from a culture on maintenance medium, and growth permitted for 24 hr. at 37° C. From this broth culture, 1 ml. was inoculated into a 1 in. diameter test-tube containing 27 ml. of the following medium: (1)–(5), as above; (6) distilled water to 1 l.; pH 7·0–7·2; autoclaved at 10 lb./in.² for 20 min. and 2 ml. of 44 per cent glucose monohydrate (autoclaved at 15 lb./in.² for 20 min.) added aseptically to each 25 ml. and the whole then steamed for 20 min. After incubation at 37° C. for 24 hr., 1 ml. of this culture was inoculated into a 250 ml. conical flask containing 50·4 ml. of lactate-asparagine medium composed as follows: (1)–(5), as above; (6) $MnSO_4$, $4H_2O$, 1 ml. 0·3 per cent solution; (7) distilled water to 1 l.; pH 7·0–7·2; autoclaved at 10 lb./in.² for 20 min. and 0·4 ml. lactate solution (prepared by dissolving 56 g. lactic acid "Analar" in distilled water, neutralizing with NaOH, making up to 250 ml. and autoclaving at 15 lb./in.² for 15 min.) added aseptically to each 50 ml. and the whole then steamed for 20 min.

Growth.—The flask was incubated at 37° C. for 4 days (though the period of incubation could be extended to 8 days or even more without loss of potency). A thin surface

film formed in the first 24–28 hr. and later disintegrated. To harvest, the whole culture (pH usually about 8·8) was acidified to approximately pH 2·5 with conc. HCl and autoclaved in its flask at 10 lb./in.^{2} for 10 min., and the crude liquor, or its supernatant after centrifuging, assayed and used for some of the experiments. It usually contained 300–600 units/ml. (see method of assay, below).

At temperatures of 25° C. and 56° C. little growth was obtained on the above medium; but with the addition of Difco yeast extract at 0·6 per cent, good growth and production of antibiotic resulted at 25°.

Larger scale production.

Inoculum.—A spore-suspension was prepared as follows: *B. licheniformis* was grown on 30 ml. of maintenance medium (see above) in 8 oz. "medical flats" for 7 days at 37° C. to complete sporulation. The spores were washed off with distilled water and diluted to give a suspension of opacity 40–50 (Brown's standards). This suspension was heated for 90 min. at 60° C. to destroy vegetative cells, and the viable count (3–6 × 10^9) was determined on tryptic beef digest agar, using the surface-plate technique of Miles and Misra (1938). The suspension was stored in the refrigerator.

Medium.—For larger scale production the medium used by Callow and Hart (1946) had the disadvantages that asparagine was required, that the concentration of glucose was critical, and that large amounts of slimy carbohydrate material were formed. Subsequently (Hart and Hills, 1947) the lactate-asparagine medium, which avoided the use of glucose, was devised, and then an ammonium and sodium lactate medium, with no asparagine, which was suited for larger scale production since it was inexpensive and, being a "poor" medium, discouraged the growth of contaminants. The latter medium comprised: K_2HPO_4, 1 g.; sodium citrate, 0·5 g.; $MgSO_4$, $7H_2O$, 1 g.; sodium lactate (50 per cent w/w), 10 ml.; ammonium lactate ("syrupy," B.D.H., or "50 per cent," Hopkins and Williams), 10 ml.; $MnSO_4$, $4H_2O$, 1 ml. of 0·3 per cent solution; iron ammonium citrate (scales), 1 ml. of 1·7 per cent solution; distilled water to 1 l. This was brought to pH 7·0 (1–2 ml. conc. HCl for 10 l.) and autoclaved at 15 lb./in.^{2} for 15 min. After autoclaving it had usually pH 6·5–6·7.

Growth.—Ammonium and sodium lactate medium, distributed in layers of 10 mm. average depth in lidded 25 × 40 cm. enamelled trays holding about 1 l., was inoculated with about 10^8 spores/l. The trays were incubated at 37° C. for 6 days. The whole culture (usually now at pH 8·8–9·6) was brought to pH 2·5 with HCl and boiled for 20 min. or autoclaved at 10 lb./in.^{2} for 10 min. The liquor obtained after passing through a high-speed continuous centrifuge was assayed and was used for processing; it usually contained 400–600 units/ml.

In the first stages of this investigation, with small cultures grown for short periods (e.g. 2–4 days), it was found that, like diplococcin (Oxford, 1944), active material was liberated from the bacteria by heating with dilute acetic acid. The process of autoclaving after bringing to pH 2·5 with HCl had the same effect. It was then found that after longer periods of growth active material was present free in the medium. In the larger scale process now described it is probable that all but a small proportion of the active material was free, and the main advantage of acidification and heating was sterilization, and coagulation of the solid material.

Extraction.—The clear liquor was brought to pH 5 by addition of 12 N NaOH solution. A slight precipitate formed which was removed by passing through a high-speed continuous centrifuge, and the supernatant fluid was run on to a bed of adsorbent carbon. For a 300 l. batch this was formed in an earthenware vacuum filter on a 24 in. plate, covered with milk-filtering muslin, by running on a slurry of 2 kg. of kieselguhr, which was allowed to drain and then covered with a slurry of a mixture of 2 kg. of kieselguhr and 2 kg. of carbon. This was allowed to drain and consolidated by gentle patting. The kieselguhr used was Johns-Manville "Hyflo Super-Cel," and the carbon, Grade

No. 111, was supplied by Messrs. Sutcliffe, Speakman & Co. In orienting experiments this carbon was found to be superior to grades available from chemical suppliers in ease with which active material could be eluted. The active liquor was first baled on in small portions to avoid disturbing the carbon surface. Slight suction (10 cm. Hg) was applied at first, giving a rate of filtration of about 50 l./hr. Filtration could be completed with suction, or the fluid allowed to drain through unaided. The last runnings were tested and found to contain little or no activity unless accidental breakage of the filter bed had occurred. The filter bed was then washed successively with 20 l. water and 40 l. industrial methylated spirit (" Methcol " : 95 per cent ethanol, 5 per cent methanol) and sucked just dry.

The eluting fluid consisted of an acid, saturated aqueous solution of *n*-butanol prepared by adding 20 vol. conc. HCl and 100 vol. *n*-butanol to 880 vol. water. This was run gently on to the filter bed and allowed to drain through under its own weight. The first runnings of displaced spirit were discarded. Collection was begun when the filtrate, after neutralizing, gave a detectable precipitate with saturated aqueous picric acid solution, and was continued until this reaction was no longer given. In different batches, from 40 to 60 l. of eluate were collected. Eluent containing half the amount of HCl was also used, but elution appeared to be slower.

The yield of activity obtained in the eluate varied rather widely ; the average was about half that present in the original medium. Three methods were tried for further concentration. Evaporation could be carried out under reduced pressure at about 50° C. without much loss, either with acid or with neutralized solutions, but was inconvenient. Precipitation of the neutralized solution by saturated methyl orange (sodium helianthate) solution was also troublesome. Re-adsorption and elution, although it entailed loss of active material, was preferred to the above processes. The eluate was first brought to pH 8 by addition of NaOH, and the flocculent precipitate removed by filtration. The pH was then brought to 6 with HCl and the solution (50 l.) run through a filter bed of 250 g. kieselguhr supporting a mixture of 250 g. kieselguhr and 250 g. carbon on a 12-in. filter. The filter was eluted with 10 l. 3 N HCl to which 0·1 vol. of *n*-butanol had been added. The eluate was brought to pH 6 and poured into an excess of picric acid solution (450 g. in 50 l. water). A little filter-paper pulp was added and the precipitate collected on a Buchner funnel. The picrate was decomposed by grinding with successive quantities of 100, 100, and 50 ml. 6 N HCl, filtering each time, and the combined filtrates were poured into 5 l. acetone. The gummy precipitate settled overnight and was then dissolved in a little methanol and the solution poured into 2 l. acetone. A white, solid precipitate was obtained which was collected on a sintered glass funnel and dried *in vacuo*. This material was obtained in a yield of 8–10 g. from a 300 l. batch of medium. The overall yield of activity averaged 25 per cent.

Batches of this hydrochloride had potencies of 2·5–5 $\times$ 10^6 units/g., based on the test strain of *Mycobacterium phlei* (see assay method, below). They were used for most of the experimental work.

Assay of the Active Material.

Solutions for assay were previously sterilized by bringing to pH 2·5 with conc. HCl (solids being dissolved in dil. HCl at this pH) and autoclaving (10 lb./in.^{2} for 10 min.). Serial dilutions of the test solution were made in portions of Hartley digest broth (pH 7·6) in 6 $\times$ 5/8 in. test-tubes (starting with 1/10 or 1/20 dilutions, in which the pH of the broth was not significantly affected by the pH of the test solution) ; and the standard test organism—a laboratory strain of *M. phlei*—was sown on the surface, using a very small circular inoculum (e.g. 1 mm. in diameter) taken from the surface of a broth culture. The assay broths were incubated at 37° C. and read after 4 days, by which time the film in the control tube had usually covered the entire surface. The highest dilution with complete inhibition was taken as end-point, and the reciprocal of this

figure was taken as the number of units in 1 ml. of the test-solution; from this the number of units in 1 mg. of a solid preparation under test could be calculated. Thus, (1) crude liquor giving inhibition up to 1 : 640 dilution would contain 640 units/ml.; (2) a solid concentrate, dissolved at 0·25 mg./ml., which gave inhibition up to 1/640 dilution, would contain $640 \times 1000/0{\cdot}25 = 2{,}560{,}000$ units/g. Since there is some variation in end-point, for accurate assays it is necessary to use small steps in the serial dilution, to carry out the tests at least in triplicate, and to compare with a standard solid preparation. In the course of the work, one of the most active preparations obtained by the method described above was set aside as a standard; the average potency of 5×10^6 units/g. (1 unit = 0·2 μg.) was taken as a fixed value and estimations of activity of unknown materials carried out in comparison with this standard.

PROPERTIES.

Physical and Chemical Properties.

General.

Certain qualitative observations made on culture fluids and crude preparations throw some light on the general properties of the active material and on the rationale of the methods of extraction adopted in the early and later stages of the investigation.

A trial was early made with treatment of cultures with acetone, but activity was divided between the supernatant and the precipitate. Attempts to extract activity from cultures at pH 6·4–6·5 with *n*-butanol were unsuccessful. Subsequent experiments with purified material showed that no activity was extracted from aqueous solutions by *n*-butanol, chloroform, ether, or ethyl acetate, over a range of pH between 1 and 9. Precipitates obtained by treatment of cultures, or of supernatants of autoclaved cultures, with ammonium sulphate contained little activity.

The method of preparing a crude concentrate by precipitation with ethanol at pH 2·7, described by Callow and Hart (1946), is evidently to be explained by adsorption on a precipitate of polysaccharide material formed in this medium, the adsorption being rather more complete at pH 2·7 than at pH 5 or 7. From solutions of this precipitate active material was precipitated by ammonium reineckate, and the polysaccharide, devoid of activity, was recoverable by precipitation with acetone. (The latter was an amorphous material having $[\alpha]_D$ about $+ 60°$ in water; it was not further investigated). The reineckate was evidently a complex material in which activity was spread over fractions soluble or insoluble in acetone. It could not be decided whether this phenomenon was due to adsorption or to the occurrence of active material in more than one form.

Attempts to use base-precipitants directly on supernatants of autoclaved cultures failed for various reasons. Picric acid gave colloidal suspensions and only partial precipitation under favourable conditions. Nitrobarbituric acid precipitated about half the active material, but fractionation of the precipitate was not possible. Rufianic acid gave a highly insoluble and inactive precipitate from which active material could not be recovered. Attempts at separation by way of the phosphotungstate or bismuth iodide compound gave materials difficult to handle and entailed much loss of activity.

A series of orienting experiments on adsorption of activity by charcoal from solutions of alcohol-precipitated material showed that some activity remained in

the solution at pH 1 and 2·2, but that removal was virtually complete at pH 5–9. Various eluents were tried, including methanol with addition of 0·5–4 per cent by vol. of conc. HCl (2 per cent gave the highest yield), methanol + NaOH, methanol + H_2SO_4, dilute acetic acid, and, finally, acid aqueous *n*-butanol solution, suggested by the process in use by Messrs. Glaxo Ltd. for penicillin. Several grades of adsorbent carbon were also tried, and the effect of "poisoning" the carbon by cyanide and of washing it with dilute acetic acid was investigated. Some improvement in elution was obtained after these pre-treatments on occasion, but it was in no case marked, and no effect appeared to be produced with the grade of charcoal finally adopted.

Filtration through bacteriological filters and dialysis.

The proportion of active material passing through an asbestos pad filter (Ford Sterimat SB), either from culture media or from solutions of concentrates when filtered in moderate quantities, varied both with pH and with initial concentration; low pH or high concentration was favourable, but in the neighbourhood of neutrality and at low concentration, almost all of the activity was held back. Thus, using pads of 3·5 cm. diameter and about 20 ml. fluid, a solution of solid concentrate ($2{\cdot}5 \times 10^6$ units/g.) at 2560 units/ml. was reduced to 640 units/ml. activity by filtration at pH 7, whilst a solution of the same concentrate at 250 units/ml. was reduced to less than 20 units/ml. by filtration at pH 7, but remained at 250 units/ml. if filtered at pH 1·5. Similarly, a culture liquor supernatant, initially at 240 units/ml., when filtered at pH values of 9, 7, 6, 2·5 and 1·5, was reduced to activities of <5, <5, <5, 50 and 150 units/ml., respectively. Adsorbed activity could be recovered by elution with dilute HCl. This adsorption and elution could not be applied to separation of active material, for the proportion adsorbed was evidently small relative to the adsorbent. Filtration through a "Gradocol" collodion membrane (0·56μ porosity) took place with less loss, though even here a low pH was favourable.

A single experiment on the dialysis of a concentrate containing polysaccharide showed that active material diffused slowly through a cellulose membrane from a solution at pH 2·2. Carbohydrate could not be detected in the dialysate by the Molisch test.

Stability.

No systematic study of stability has been made, but a high degree of stability is indicated by the absence of significant loss when autoclaved at 10 lb./in.^{2} for 10 min. over the pH range 1–9. However, a solution of a purified concentrate in water at pH 7 containing 5000 units/ml. lost 75 per cent activity after 6 months at room temperature in light. In presence of concentrated acid (6 N HCl) or alkali (10 N NaOH) activity was lost completely in a period of a few days, more rapidly with the alkali. There was no evidence of deterioration of dry preparations after storage at room temperature in the light.

Physical properties.

Hydrochloride.—Licheniformin hydrochloride was obtained by the process described above as a white amorphous powder, which did not melt but charred on heating strongly. Although crude preparations were deliquescent, this pro-

perty was lost on purification. It was extremely soluble in water and very soluble in methanol, but sparingly soluble in ethanol. It was insoluble in dry *n*-butanol, acetone and non-polar organic solvents.

The following values for specific optical rotation in aqueous solution were obtained. (i) Unpurified standard preparation of hydrochloride: $[\alpha]_D$–37°; $[\alpha]_{5461}$–44° (c = 1·09 per cent). (ii) Material (i) after conversion to helianthate, recrystallization and reconversion to hydrochloride: $[\alpha]_D$–37°; $[\alpha]_{5461}$–44·5° (c = 1·24 per cent). (iii) A fraction of material eluted by aqueous ethanol after adsorption from ethanolic solution on a chromatograph column of acid-washed alumina: $[\alpha]_D$–37·5°; $[\alpha]_{5461}$–47° (c = 0·93 per cent).

Other salts.—The sulphate, obtained from the picrate by treatment with dilute sulphuric acid and precipitation by acetone, was obtained as a white powder consisting of glassy particles. The helianthate was prepared by adding 1 l. cold, saturated, aqueous solution of methyl orange to 100 ml. neutralized solution of 1 g. licheniformin hydrochloride. The orange-red precipitate was recrystallized from 500 ml. hot 33 per cent aqueous methanol, crystallization of the initial oily precipitate being induced by scratching. The product was a red powder consisting of microscopic needles which decomposed when heated. The reineckate was obtained as a crystalline precipitate, but could not be recrystallized successfully. The rhodanilate was oily. The picrate, picrolonate, and flavianate were obtained as oily, semi-solid precipitates, the rufianate as an amorphous solid, and the oxalate as an amorphous solid by way of the helianthate. None of these could be crystallized.

Colour reactions.

Positive reactions were obtained with the biuret test for the amido group and with the Sakaguchi test for the guanidino group. No positive reaction was obtained with the ninhydrin test for α-amino acids, the Molisch test for carbohydrates, the test of Elson and Morgan (1933) for glucosamine, or the modified test of Scudi, Boxer and Jelinek (1946) for glucosamine (given by streptomycin). A peculiar reaction is the formation of a precipitate on the addition of 1 per cent sodium hypochlorite solution to the aqueous solution.

Elementary analysis.

The following figures were obtained: (i) Unpurified standard preparation of hydrochloride: C, 41·5; H, 7·5; N, 15·7; Cl, 9·5 per cent. (ii) Hydrochloride from recrystallized helianthate: C, 39·6; H, 7·6; N, 17·3; Cl, 9·8 per cent. (iii) Fraction eluted from acid-washed alumina: C, 41·0; H, 7·5; N, 17·4; Cl, 8·4 per cent. (iv) Sulphate: S, 8·75 per cent. In determination by the Van Slyke method a prolonged evolution of nitrogen took place, equivalent to the following percentages of amino nitrogen after the times stated: 5 min., 1·8; 15 min., 3·0; 30 min., 3·4; 45 min., 3·5.

Antibacterial Properties in vitro.

Effect of inoculum size.

The effect of increasing the size of the inoculum on the antibacterial titre could not be ascertained satisfactorily using surface culture of *M. phlei*, but a depressing

effect was markedly evident with many organisms grown in subsurface culture, as shown in Table I for a streptococcus.

TABLE I.—*Relation of Titre of Activity to Inoculum Size.*

Serial twofold dilutions, in 4 ml. portions of digest broth, of licheniformin HCl, potency 5×10^6 units/g. Test organism : *Str. pyogenes* T24 (Group A). Inocula : Various volumes of overnight broth culture.

Inoculum (ml.).	Dilution inhibiting growth (1 in ... thousand).		
	18 hr.	48 hr.	96 hr.
0·1	40	20	20
0·002	640	160	160
0·001	1250	320	320
0·0002	>2500	1250	640

Effect of serum.

This effect likewise could not be studied using *M. phlei*, owing to inhibition of its growth in the presence of 25 per cent or more of serum. With a *Staph. pyogenes* strain as test organism, no inactivation was apparent. Thus, 0·5 ml. licheniformin solution (300 units/ml.), incubated overnight with 0·5 ml. mouse serum and then serially diluted in broth, gave the same anti-staphylococcal titre as when treated similarly with 0·5 ml. water. Again, serial dilutions of a solid preparation in broth containing 50 per cent horse serum gave the same end-point against another strain of *Staph. pyogenes* at 48 hr. as did a similar series in plain broth.

Effect of pH.

The reaction of the medium in which antibacterial tests were carried out had a marked effect upon the end-point, as shown in Table II.

TABLE II.—*Effect of pH on Activity.*

Serial twofold dilutions of crude culture liquor, potency 640 units/ml., in digest broth at various pH values. Test organism : Rapidly growing *M. tuberculosis* (A.T.C.C. 607), surface culture.

	pH of broth.							
	8·5	8·0	7·8	7·1	6·8	6·2	6·0	5·5
Growth inhibited in dilution of 1 in	1280	640	640	320	240	80	40	10

A similar relationship between titre and pH has been shown for streptomycin, also a basic substance, and contrasts with the behaviour of penicillin (see Abraham and Duthie, 1946).

Range of susceptible bacteria.

The growth of a fairly wide range of bacteria, more especially of Gram-positive species, was inhibited, as is shown in Table III. The inoculum was small in most cases, and the significance is largely relative. High or moderate activity

was evident against a number of strains of pathogenic *Corynebacteria*, and of *Staph. pyogenes*, *Str. pyogenes*, *M. tuberculosis*, *Pasteurellae*, *Brucella abortus* and *B. anthracis*. Against *Shig. dysenteriae* and *E. coli* the drug was less active. A small point of interest is the wide difference in sensitivity between certain corynebacteria met with in veterinary practice; *C. equi* and *C. renale* were highly sensitive, while *C. pyogenes* and *C. ovis* were resistant.

TABLE III.—*Antibacterial Activity of Licheniformin against Various Organisms.*

Licheniformin HCl, potency $2{\cdot}5$–$5{\cdot}0 \times 10^6$ units/g. Method: Serial twofold dilutions in 4 ml. Hartley digest broth. Inoculum: Overnight broth culture 0·001–0·0001 ml./tube. Reading: after 18 hr. at 37° C.

Organism.	Highest dilution giving inhibition of growth (1 part in . . .).
Corynebact. renale (1)*	4×10^7†
Corynebact. diphtheriae (6)	10^7–$1{\cdot}25 \times 10^6$†
Corynebact. equi (1)	5×10^6†
Mycobact. phlei (1)	5×10^6–$2{\cdot}5 \times 10^6$‡
Staph. pyogenes (5)	5×10^6–$1{\cdot}25 \times 10^6$
Str. groups A, B, C (13)	$1{\cdot}25 \times 10^6$–8×10^4
Mycobact. tuberculosis (4)§	$6{\cdot}4 \times 10^5$–$1{\cdot}6 \times 10^5$‖
Past. septica (7)	$6{\cdot}4 \times 10^5$–$<5 \times 10^3$
Brucella abortus (3)	$3{\cdot}2 \times 10^5$¶
B. anthracis (1)	2×10^5**
Shig. dysenteriae Shiga, Flexner *V*, *Z* (3)	$1{\cdot}6 \times 10^5$–4×10^4
Dip. pneumoniae (3)	$1{\cdot}6 \times 10^5$–2×10^4
E. coli (5)	8×10^4–10^4
Ps. pyocyanea (1)	4×10^4
V. cholerae (1)	2×10^4
Str. viridans (1)	2×10^4
Salm. paratyphi B (2)	2×10^4–10^4
Actinobacillus lignieresi (2)	$<10^4$
Erysipelothrix rhusiopathiae (2)	$<5 \times 10^3$
Corynebact. pyogenes (3)	Low activity.
Corynebact. ovis (1)	,,

*Bracketed figures denote numbers of strains tested.
† Read at 72 hr.
‡ Surface inoculum; read at 96 hr.
§ The four strains were virulent. They were human types H37 Rv (from the American depot at Trudeau Sanatorium, Saranac Lake), "Pickup" (a stock strain maintained at these laboratories) and "Gambie 11" (recently isolated from a patient's sputum); and bovine type SB (a stock strain from these laboratories).
‖ Modified Dubos albumin-"Tween 80" synthetic medium; inoculum of 0·04 mg. (moist weight) in 5·5 ml. medium containing antibiotic, in 25 mm. diam. test-tubes; read at 14 days.
¶ Read at 96 hr.
** Inocula of 2×10^4–$1{\cdot}6 \times 10^5$ spores/ml. (Dr. J. M. Barnes).

Characters distinguishing licheniformin from certain other antibiotics.

A number of antibiotics have been recently described which are derived from "*B. subtilis*," a group related to, and by some authors not differentiated from, *B. licheniformis*. In some cases a sufficiently full description has been given for

it to be clear that they differ from licheniformin in at least one salient character. Thus subtilin (Jansen and Hirschmann, 1944) is sparingly soluble in dilute sodium chloride at acid reaction and contains 4·2 per cent of sulphur (Dimick, Alderton, Lewis, Lightbody and Fevold, 1947). Serum is strongly antagonistic *in vitro* to bacillin and this substance is inactive *in vivo* (Foster and Woodruff, 1946). Endosubtilysin is extractable from the medium at pH 3 by chloroform (Olivier, 1946; De Saint-Rat and Olivier, 1946). Bacitracin is extractable by butanol (Johnson, Anker and Meleney, 1945). In other cases the physical and chemical properties have not yet been sufficiently defined to permit of comparison with licheniformin. These substances include subtilina (Fontes Magarao, Arriagada and Thales, 1944), subtiline (Ramon, Richou and Ramon, 1945), eumycin (Johnson and Burdon, 1946) and colistatin (Gause, 1946).

As streptomycin is extracted by a method similar to that used for licheniformin, attention was particularly directed to distinguishing these two materials. They differ in elementary composition, and licheniformin does not give the reaction of Scudi, Boxer and Jelinek (1946), nor does it appear to yield maltol or streptidine. A clear distinction is also afforded by the relative inhibitory activity of the two antibiotics against the standard strain of *M. phlei* and a bovine strain (SB) of *M. tuberculosis*, the appropriate conditions of test on one organism being the same for both antibiotics. The results are shown in Table IV.

TABLE IV.—*Differentiation of Licheniformin from Streptomycin on Basis of Antibacterial Activities.*

Licheniformin HCl, potency 5×10^6 units/g., and streptomycin HCl, potency 690 μg. base/mg. Cultural conditions: For *M. phlei*, surface culture in Hartley digest broth; for *M. tuberculosis*, deep culture in modified Dubos albumin-Tween synthetic medium.

	Highest dilution giving inhibition of growth.	
	Licheniformin HCl.	Streptomycin (base).
(a) *M. phlei*	$1 : 10^7$	$1 : 8 \times 10^5$
(b) *M. tuberculosis*	$1 : 1{\cdot}6 \times 10^5$	$1 : 3{\cdot}5 \times 10^6$

Antibacterial activity of other strains of B. licheniformis, *and of other aerobic spore-forming bacilli.*

Eleven strains of *B. licheniformis* (ten from the National Collection of Type Cultures) were tested for antibiotic production by the small scale method described on p. 418, using the same lactate-asparagine medium. In each case the acidified and heated culture liquor was active in varying degree against the standard *M. phlei* and, where tested (7 strains), also against *M. tuberculosis* SB. By contrast, 70 strains of other species of aerobic spore-forming bacilli (also from the National Collection), including *B. subtilis*, *B. mesentericus* and *B. megatherium*, were similarly assessed, but in none of the cultures was there evidence of activity against *M. phlei*; some of the strains grew poorly on the medium used. Among the strains not productive under these conditions was N.C.T.C. 7241 (= A.T.C.C. 6633 = N.R.S. 231), a strain of *B. subtilis* reported to be a producer of subtilin (see above), and this has also been found with N.R.R.L.-B. 543, a subtilin-pro-

ducing strain recently received from Dr. H. Lineweaver of the Western Regional Research Laboratory.

Under the conditions of culture used, this striking limitation of licheniformin-like activity to strains identified as *B. licheniformis* would appear to support the distinction made on other grounds between this species and *B. subtilis* (Gibson, 1944).

Pharmacological Properties.

Toxicity of single injections to mice.

For injection, licheniformin hydrochloride was dissolved in water or normal saline and the solution brought to pH 6·5–7·5. The lethal doses after single injections are shown in Table V.

TABLE V.—*Acute Toxicity of Licheniformin to Mice.*

Mice 18–20 g. wt. Licheniformin HCl nine batches, potency 2·5–5·0 $\times 10^6$ units/g. Injection volumes 0·4 ml.

Dose (mg.).	Survivors at 7 days (aggregate of experiments).		
	Intravenously.	Intraperitoneally.	Subcutaneously.
1·25	10/10	13/13	4/4
2·5	14/14	19/19	6/6
5	11/17	17/21	8/8
10	2/15	3/22	5/8
20	0/4	0/18	2/8

The LD_{50} of these preparations were thus approximately 5 mg. intravenously, 7·5 mg. intraperitoneally and 10 mg. subcutaneously; and the corresponding LD_0 were 2·5 mg., 2·5 mg. and 5 mg. A comparative series of experiments with two specimens of streptomycin (Pfizer lot P 4612, potency 800 μg. base/mg., and Merck lot 527, 650 μg./mg.) gave values for LD_{50} of about 2·5 mg. intravenously, 15 mg. intraperitoneally and 15–20 mg. subcutaneously, and corresponding values for LD_0 of 1·25 mg., 10 mg. and 10 mg. (weights of active base).

It follows that, weight for weight, the licheniformin preparations were less toxic than streptomycin intravenously, but more toxic than the latter drug by the other routes. The difference between toxicity by intraperitoneal or subcutaneous administration and that by intravenous injection was thus small for licheniformin, but marked for streptomycin. Death after intravenous injections of licheniformin occurred usually within 1–2 hr., and after the large doses within a few minutes. After fatal injections by the intraperitoneal or subcutaneous routes, death took place usually within 24 hr.; if a mouse survived this period it rarely died later during the week of observation.

Toxicity of repeated injections to mice.

There was a low mortality (15 per cent) in mice injected subcutaneously with 6 mg. daily (2 mg. mornings, 4 mg. evenings) for 5–6 weeks, but survivors were in poor condition; at autopsy the most striking change was in the kidneys, which were enlarged and pale and microscopically showed glomerular-tubular damage. At a reduced daily dose of 1·5 mg. (0·5 mg. mornings, 1·0 mg. evenings) for a

similar period, the mortality was less than 5 per cent and the animals remained in fairly good condition; but the renal changes, while distinctly less, were still evident.

Effect on blood.

Licheniformin hydrochloride (4×10^6 units/g.) was dissolved in normal saline to make a 0·5 per cent solution, and washed horse red cells were added to a final concentration equivalent to 1 per cent original blood. No appreciable haemolysis was evident after incubation overnight at 37°C. Larger concentrations of the drug were not tested.

The effect on phagocytosis by white blood cells was examined by Dr. F. C. O. Valentine. Licheniformin was added to blood and the mixture left for 3 hr.; killed staphylococci were then added and films made after 30 min. Phagocytosis was normal in the presence of 0·5 per cent licheniformin, but was prevented by 1·0 per cent. If, however, the cells were washed in saline after the 3 hr. contact with the drug, even at 5·0 per cent, active phagocytosis took place when fresh serum and staphylococci were added.

Tests *in vitro* (Dr. Valentine) suggested that licheniformin tended to prevent the formation of a firm blood clot. Furthermore, a delay in clotting of the heart blood was observed by us in mice that had received fatal injections of the drug.

Other pharmacological reactions.

Tests made by Dr. F. C. MacIntosh and Dr. W. D. M. Paton showed that in doses of 1–2 mg./kg. samples of licheniformin produced a transient sharp fall in the blood pressure of cats under influence of chloralose. This effect is considered to be due to the liberation of histamine from skin and muscle by the drug. Large doses (10–20 mg./kg.) produced a shock-like condition. Intracutaneous injection caused a typical "triple response" in human skin in dilutions down to 10^{-5}. The drug had no action on the isolated ileum of the guinea-pig. This work will be reported in detail elsewhere.

Blood levels in mice after subcutaneous administration.

The levels of licheniformin in the blood of mice after varying subcutaneous doses were followed, using a broth-dilution method of assay, with *Staph. pyogenes* as test organism. The levels reached a maximum at 1–2 hours and then fell away. Comparison is made with streptomycin in Fig. 1. Bearing in mind that the nature of the unit differs for the two drugs, it can be seen that the maximum level is reached later after injecting licheniformin and that the latter drug persists rather longer in the blood.

Protective Effects against Certain Experimental Infections in Mice.

On the basis of the results shown in Table III, protective experiments were carried out in mice on infections by staphylococci, streptococci, anthrax and tubercle bacilli.

Staph. pyogenes.

The details of the main experiment (Mrs. J. Bedford) are shown in Table VI.

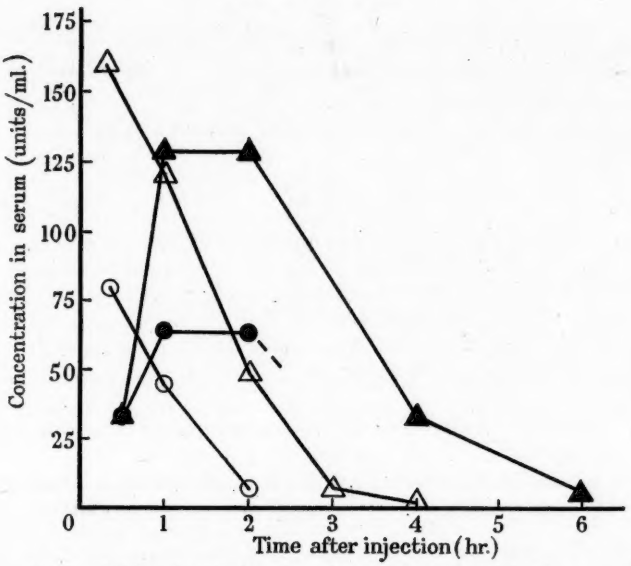

FIG. 1.—Serum levels of licheniformin in mice after subcutaneous injection compared with the results of Rake and Donovick (1947) for streptomycin.

△ Streptomycin 250,000 units/kg. ▲ Licheniformin 375,000 units/kg.
○ Streptomycin 104,000 units/kg. ● Licheniformin 125,000 units/kg.

TABLE VI.—*Mouse Protection Test against* Staphylococcus pyogenes *Infection.*

Mice, albino Parkes strain,* wt. 20 g. Intraperitoneal infection with 0·5 ml. of various dilutions of an 18 hr. broth culture of licheniformin-sensitive *Staph. pyogenes* (containing approx. 340×10^6 organisms/ml.) plus 0·5 ml. sterilized mucin. Licheniformin HCl, $2{\cdot}5 \times 10^6$ units/g., given subcutaneously in 0·25 ml. water pH 6–7, thrice on 1st, twice on 2nd and twice on 3rd days, starting 1 hr. after infection. Surviving animals killed at 17 days.

Dilution of infecting culture. (1 in ...)	Drug. Single dose. (mg.)	Drug. Total dose. (mg.)	Mice (original number).	Deaths each day. 1.	2.	3.	4.	5–10.	11–17.	Total deaths.
Undiluted	5	35	3	2	0	0	1	0	0	3
	0·5	3·5	3	3	0	0	0	0	0	3
	—	—	6	6	0	0	0	0	0	6
10^1	5	35	3	0	0	1	1	1	0	3
	0·5	3·5	3	0	1	0	0	2	0	3
	—	—	6	6	0	0	0	0	0	6
10^2	5	35	3	1	0	0	0	0	1	2
	0·5	3·5	3	0	0	0	0	0	1	1
	—	—	6	2	2	1	0	1	0	6
10^3	—	—	6	1	1	0	0	3	1	6
10^4	—	—	6	0	0	0	0	3	3	6

* This is a line-bred strain maintained at the National Institute for Medical Research, Mill Hill.

After the weaker infecting doses the drug prolonged life, but the treated animals tended to die in the later periods and these mice, as well as the survivors killed at 17 days, showed internal abscesses at autopsy. There was thus a definite chemotherapeutic effect, but not a radical one. As judged on the small number of animals, there was no appreciable difference between the two levels of drug dosage.

Str. pyogenes.

The wide range of variation in susceptibility of strains of *Str. pyogenes* to licheniformin *in vitro* is evident from Table III. It is not surprising, therefore, that the antibiotic failed to protect mice against a strain of Group A, Type III, whose sensitivity was at the lower end of the range. On the other hand, the Aronson strain of Group B, which was considerably more sensitive to licheniformin *in vitro*, gave the results shown in Table VII.

TABLE VII.—*Mouse Protection Test against Infection with Sensitive Strain of* Str. pyogenes.

Mice infected intraperitoneally with 0·5 ml. of various dilutions of a 24 hr. mouse-blood culture. Licheniformin HCl, 2·5–5·0 × 10^6 units/g., given subcutaneously thrice on 1st, twice on 2nd, and once on 3rd day, starting 1 hr. after infection; single dose 3 mg. in 0·5 ml. water pH 6–7; total dose 18 mg./mouse. Survivors killed at 8 days.

Dilution of infecting culture. (1 in ...)	Category.	Mice (original number).	Deaths each day.								Total deaths.
			1.	2.	3.	4.	5.	6.	7.	8.	
10^5	Treated	6	0	0	0	0	1	2*	0	0	3
	Control	6	6	0	0	0	0	0	0	0	6
10^6	Treated	6	0	1	1	0	0	0	0	0	2
	Control	6	3	3	0	0	0	0	0	0	6
10^7	Treated	6	0	2	0	0	1	0	0	0	3
	Control	6	0	6	0	0	0	0	0	0	6
10^8	Control	6	0	2	0	0	0	0	0	0	2

* Streptococci recovered from heart blood.

There was thus some prolongation of life, and a higher survival incidence at the end of the experiment in the drug-treated group than in the controls, but the effect was not of a high order.

B. anthracis.

An experiment (carried out by Dr. J. M. Barnes, Microbiological Research Department, Experimental Station, Porton) is detailed in Table VIII. Licheniformin, at two levels of dosage, was compared with streptomycin and with penicillin, to each of which the strain of *B. anthracis* used was sensitive. The anthrax spores were given intraperitoneally and the drugs subcutaneously (Barnes, 1947).

A considerable and radical protective effect was shown by each of the three antibiotics as compared with the controls receiving saline. It might be justifiable to conclude that 1·5–9 mg. of licheniformin hydrochloride, 0·9 mg. of strepto-

TABLE VIII.—*Mouse Protection Tests against* B. anthracis *Infection.*

Mice: Swiss strain 17–20 g. Intraperitoneal infection with 0·1 ml. suspension containing 4×10^4 spores/ml. Licheniformin HCl, $2{\cdot}5–5 \times 10^6$ units/g.; streptomycin HCl (Boots), 620μg. base/mg.; penicillin (Glaxo), $1{\cdot}6 \times 10^6$ units/g.; each injected subcutaneously in the doses stated at 4, 6 and 8 hr. after infection; injection vol., 0·3 ml. Dead animals, and survivors killed at 8 days, were autopsied.

Drug.			Mice (original number).	Deaths each day.							Deaths.	
Name.	Single dose.	Total dose.		1.	2.	3.	4.	5.	6.	7.	Total.	Percentage.
Licheniformin	3 mg.*	9 mg.	37	0	0	2	3	0	0	1	6	16·2
,,	0·5 mg.*	1·5 mg.	40	0	0	4	4	4	1	0	13	32·5
Streptomycin	0·3 mg.†	0·9 mg.	39	0	0	1	1	2	2	0	6	15·4
Penicillin	100 units	300 units	40	0	0	0	7	1	1	1	10	25·0
Saline	0·3 ml.	0·9 ml.	37	0	12	7	13	3	0	0	35	94·5

* Refers to weight of solid. † Refers to weight of active base.

mycin base and 300 units (0·18 mg.) of penicillin had a similar order of effectiveness. A difference in protection was evident in the two dose levels of licheniformin.

M. tuberculosis.

Method.—The special chamber designed for the exposure of small animals to aerosol mists, which was based on the pilot model described by Glover (1944), is described in detail elsewhere (Bourdillon and Glover, 1948). The fully virulent bovine strain SB (which was the least sensitive to licheniformin of the 4 strains of *M. tuberculosis* mentioned in Table III) was used. After growth on egg-yolk agar, it was suspended in normal saline solution and was projected into the box as a fine spray of droplet nuclei not exceeding 5μ in diameter. The mice (females of Swiss strain, 18–20 g. wt.), housed in wire cages, were exposed to the mist for one hour. The number of organisms in the mist had been ascertained in previous similar experiments by withdrawing a measured volume of air with the micro-slit sampler (as described by Schuster, 1948) and inoculating guinea-pigs with serial dilutions of the organisms thus collected (Glover, 1944, 1948). In the present series of experiments, the estimated number of bacilli inhaled by treated animals and controls was either 10^5 or 10^6, with additional, graded, controls receiving lower doses. After exposure, the mice were grouped into "treated" and "controls," and were then housed three in a cage and fed upon a normal stock diet, or, in the sulphone experiments, upon cubed meal diet (Parkes, 1946).

The licheniformin hydrochloride preparations had potencies of $2{\cdot}5–5{\cdot}0 \times 10^6$ units/g.; the streptomycin hydrochloride (Boots, Merck) had potencies of 620–690 μg. base/mg. Solutions for injection were made in water, those of licheniformin being brought to pH 6·5–7·5. For both drugs, subcutaneous injections were started one day after infection; the daily dose was usually divided into one-third (in 0·2 ml. water) at 9–10 a.m. and two-thirds (in 0·4 ml. water) at 4–5 p.m., but on Saturdays a two-thirds dose was given at 9–10 a.m. and on Sundays no injections were given. In the sulphone experiments, the sulphetrone (in tablets) or promin was previously mixed in bulk in the broken-up cubed meal to give the desired percentage (the admixture being assisted by first mixing the sulphone with glucose powder, the final concentration of the latter in the meal being 10 per cent); 3–4 g. of the diet were offered twice daily. Experiments were terminated about 5 weeks after infection, when survivors were killed and an estimation made of the number and size of the macroscopic pulmonary foci (see footnote to Table IX). The method of notation, and assessment of group percentage scores of lesions, have been described elsewhere (Glover, 1944). In brief, the score 7 (maximum) is assigned to mice which die with extensive lesions in both lungs before the termination of the experiment (this did not occur in the present series); 6 indicates complete con-

solidation at the completion of the experiment, and lower numbers indicate decreasing involvement, viz., 5 = 3/4, 4 = 1/2 and 3 = 1/4 consolidation, and 2 = 5 to 20, 1 = less than 5 foci in the two lungs and 0 = no lesions. The mean score of a group (aggregate score ÷ number of mice) is expressed as a percentage of its maximum possible (7) to give the "group percentage score."

Licheniformin and streptomycin (*Experiments* 1–3).—In Experiment 1 the highest daily dose of licheniformin that seemed safe on the basis of acute toxicity tests, namely, 6 mg., was administered to one group, whilst another group received a daily dose of 3 mg. of streptomycin (base), which was considered adequate on the basis of published data. The number of bacilli inhaled by each mouse in the treated and main control groups was about 10^5, and two further (graded control) groups received doses of 10^4 and 10^3 organisms respectively. The experiment was terminated between the 35th and 42nd days and the degree of pulmonary involvement is shown in Table IX.

The differences in the extent and distribution of the lesions were striking. In the main control group, receiving 10^5 bacilli, the lungs were grossly enlarged. The pulmonary tissue was completely or nearly completely replaced by firm greyish-white areas which contained large numbers of tubercle bacilli. In the graded controls, the dose of 10^4 bacilli also induced fairly severe lesions affecting from 3/4 to 1/3 of the lungs; in the group receiving 10^3 bacilli there were a few scattered lesions which were readily countable and varied from 4 to 10 discrete foci in the two lungs. In the two treated groups (10^5 bacilli), 3 mice on streptomycin and 5 on licheniformin showed no macroscopic lesions; in the rest, discrete lesions less in extent than those produced by the smallest control dose were seen. Representative smears showed small numbers of acid-fast bacilli. The group percentage scores of lesions, both in the streptomycin and licheniformin-treated groups, were 13, as against 71 in the main control category, these differences being highly significant (Table IX).

Although 11 out of 12 of the licheniformin-treated mice survived to the end of the experiment these had lost weight and were in poor condition. At autopsy the kidneys were enlarged and much paler than in the controls; microscopic sections showed serious glomerular and tubular damage. The streptomycin-treated mice, by contrast, were in relatively good condition and the kidneys apparently normal macroscopically.

In order to reduce the toxic effects of the licheniformin, Experiment 2 was undertaken in which one group of mice was given 1·5 mg. and a second group 0·6 mg. daily. In view of the significant suppression of lesions in the previous experiment, the infecting dose was raised ten-fold (10^6 bacilli). On this occasion a streptomycin group was not included. The mice were killed 29 days after infection. As a result of the reduction in the dose of licheniformin, the mice in both groups maintained their weight whilst the effect on the kidneys was appreciably reduced (see below). All the mice in the main control group showed complete or nearly complete consolidation of lungs. In neither treated group were the lesions agglomerated into large irregular masses as in the controls, but they were composed of discrete foci which affected from a quarter to a half of the lung tissue. The difference was merely quantitative, since the foci showed no macroscopic or microscopic evidence of resolution. The limited reduction in lesions due to treatment is indicated numerically by the group scores of 53

TABLE IX.—*Mouse Protection Tests against Tuberculous Infection.*

Mice infected by inhalation of known, graded doses of bacilli, then given protective drugs, singly or in combination. Mice killed after about 5 weeks, lungs examined, and lesions given a score of 0–7 in individual mice. From these individual scores a "group percentage score," i.e. the mean score of a group expressed as a percentage of its maximum possible value (7), was obtained by means of the formula $\frac{\text{aggregate of individual scores}}{\text{number of suvivors}} \times \frac{100}{7}$ (see also text).

Expt.	Drug. Group.	Drug. Daily Dose*.	Estimated bacilli inhaled.	Mice. Original No.	Mice. Survivors.†	Pulmonary involvement. Group percentage score.	Pulmonary involvement. Standard error of previous column.
1	Graded control	—	10^3	6	6	23·8	4·1
	„ „	—	10^4	6	6	61·9	4·1
	Main control	—	10^5	24	22	70·8	2·1
	Streptomycin	3 mg.	10^5	12	10	12·9	3·2
	Licheniformin	6 „	10^5	12	11	13·0	3·0
2	Graded control	—	10^4	6	6	59·5	4·1
	„ „	—	10^5	6	5	65·7	4·5
	Main control	—	10^6	24	23	81·4	2·1
	Licheniformin	1·5 mg.	10^6	12	11	53·2	3·0
	„	0·6 mg.	10^6	12	12	57·1	2·9
3	Graded control	—	10^3	6	6	26·2	4·1
	„ „	—	10^4	6	6	59·5	4·1
	Main control	—	10^5	24	23	75·8	2·1
	Streptomycin	3 mg.	10^5	12	11	14·3	3·0
	Licheniformin	1·5 mg.	10^5	12	11	45·5	3·0
	„	0·6 mg.	10^5	12	10	75·7	3·2
5	Graded control	—	10^3	6	5	22·9	4·5
	„ „	—	10^4	6	6	59·5	4·1
	Main control	—	10^5	16	16	69·6	2·5
	Sulphetrone	3% in diet	10^5	16	15	65·7	2·6
	Licheniformin	1·5 mg.	10^5	16	16	43·8	2·5
	„ Sulphetrone	1·5 mg. 3% in diet	10^5	16	15	14·3	2·6

* Streptomycin doses refer to weight of active base; licheniformin doses refer to weight of actual preparation of hydrochloride.

† In none of the drug-treated mice dying before the termination of the experiments was death apparently due to tuberculosis.

Statistical notes (by Dr. C. W. Emmens): (1) The following group differences were tested and found to be highly significant, with $P<0{\cdot}001$: Exp. 1, streptomycin 3 mg. and licheniformin 6 mg. from main control; Exp. 2, licheniformin 1·5 mg. and 0·6 mg. from main control; Exp. 3, streptomycin 3 mg. and licheniformin 1·5 mg. from main control; Exp. 5, licheniformin 1·5 mg. + sulphetrone 3 per cent from licheniformin 1·5 mg. alone, from sulphetrone 3 per cent alone, and from main control. (2) Bartlett's test was applied and showed that there was no significant heterogeneity of variance. An estimate of variance derived from the whole data was therefore used in making comparisons between groups; this did not, however, affect the answer since t tests based on readings of individual groups gave equally low values of P.

and 57, which are significantly lower than the figure for the main control group, viz. 81 (Table IX).

Since it was not clear whether the much smaller suppression evident in Experiment 2 as compared with Experiment 1 was due to the reduced dose of anti-

biotic or to the increased dose of bacilli, or to a combination of both factors, Experiment 3 was performed in which the amount of licheniformin was as in the second experiment, but the dose of bacilli was reduced to 10^5, as in the first one. A group treated with 3 mg. streptomycin base daily was included. The results (Table IX) at 34 days confirmed the markedly suppressive effect of streptomycin on the pulmonary lesions at this level, but indicated that licheniformin, whilst producing significant effect at a level of 1·5 mg. daily, was considerably less effective than at the 6 mg. level. A subsequent experiment with streptomycin showed that when administered in a dose of 0·9 mg. base daily to mice infected by inhalation of 10^5 bacilli, it was no more effective than 1·5 mg. daily of licheniformin.

The kidneys of mice treated with 1·5 mg. of licheniformin daily in these two experiments showed some enlargement and glomerular-tubular damage after 4–5 weeks, though the effect was less than at the 6 mg. daily dose level.

Licheniformin and sulphetrone (*Experiments* 4–5).—In view of the results obtained by Brownlee (1948) of the effect on tuberculous infection in guinea-pigs of streptomycin given simultaneously with sulphetrone, it seemed of interest to test the combined action of licheniformin and this sulphone, and for this purpose mice were used.

The same inhalation method of infection was adopted as in the previous experiments, the dose being 10^5 bacilli; treatment with licheniformin 1·5 mg. daily by injections, with sulphetrone mixed in the diet, or with the two together, was started on the next day. In a preliminary experiment, using 12 mice in each treated group and 24 in the main control group, and lasting 39 days (Experiment 4), the sulphetrone was given at first at a level of 4 per cent; but after 4 out of the 24 mice on the drug had died, the level was reduced to 2 per cent from the 30th day onwards. A group of mice receiving 1 per cent promin in the diet was also included for comparison. The group percentage scores of the lungs of the survivors (not tabulated) were as follows: Graded control (10^3 bacilli), 33; graded control (10^4 bacilli), 64; main control (10^5 bacilli), 76; promin, 1 per cent in diet (10^5 bacilli), 80; sulphetrone, 4 per cent and, later, 2 per cent in diet (10^5 bacilli), 74; licheniformin, 1·5 mg. daily (10^5 bacilli), 50; licheniformin plus sulphetrone (10^5 bacilli), 14.

In view of the striking effect of the combined treatment, the experiment was repeated with larger groups of mice, and in order to reduce the toxic effects of the sulphetrone the level of administration of the latter was 3 per cent in the diet throughout; this dose proved safe. The promin group was omitted on this occasion. The results at the termination of the experiment, 40 days after infection, are given in Table IX, Exp. 5. They amply confirm the previous experiment. Like promin, sulphetrone alone at the highest safe dose was quite ineffective, while licheniformin, 1·5 mg. daily, had only the limited suppressive effect recorded for this dose in the previous experiments; the pulmonary lesions were reduced in size, but considerable areas of the lungs were affected. Combination of sulphetrone and licheniformin at the same dosages, however, produced as marked a suppressive effect as had licheniformin alone at 6 mg. level and streptomycin at 3 mg. (base) level (Exp. 1). The contrast with the other groups was striking; not more than 10 foci were counted in any pair of lungs and the lesions were very small, ranging from 1–3 mm. in diameter. It should be noted that renal enlargement was present in the licheniformin-treated mice, as in previous experiments.

Fig. 2 summarizes graphically the main tuberculosis results so far described, by showing: (1) The gradation of pulmonary involvement with increasing dose of bacilli inhaled, from 10^3 to 10^6 per mouse; (2) the gradation of suppression with increasing daily dose of licheniformin from 0·6 mg. to 6 mg.; (3) the effect of combination of sulphetrone with licheniformin in reducing the dose of the latter drug required to give a very considerable degree of suppression.

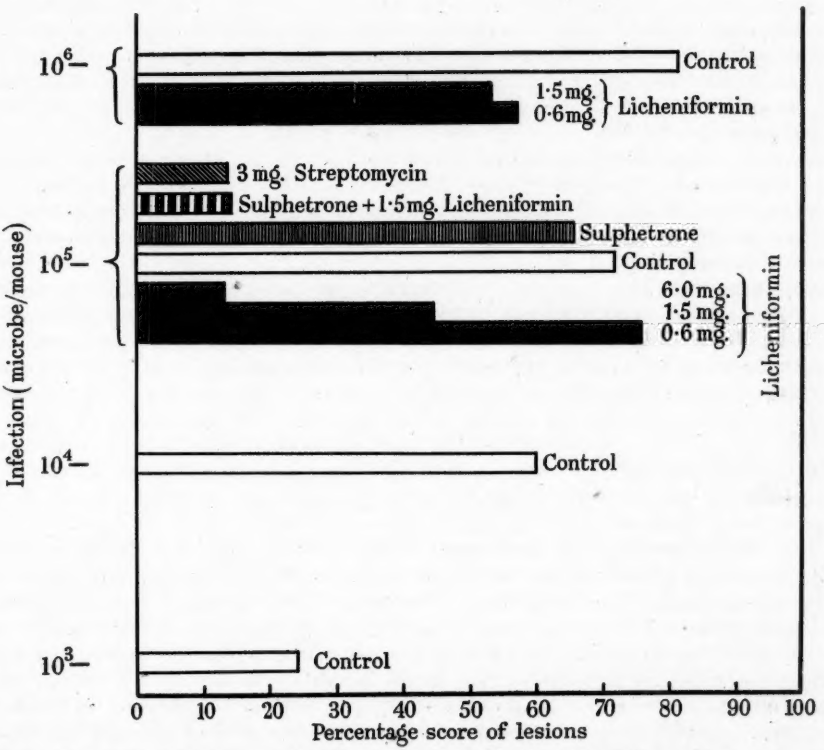

FIG. 2.—Values of percentage scores of pulmonary lesions in mice, with or without various treatments, at four levels of infection with *M. tuberculosis*. Mean values from Experiments 1–3 and 5. Weights of drugs are daily doses.

Further experiments.—In view of the synergistic effect of sulphetrone with licheniformin, Exp. 6 (not tabulated) was performed in which mice were injected with a mixture of streptomycin and licheniformin at dose levels of 0·3 mg. (base) and 0·6 mg. daily, respectively. A limited suppressive effect was noted, but this was no greater than the effects of comparable groups of mice given 0·9 mg. daily of streptomycin alone or licheniformin alone. There was thus no evidence of more than an additive effect, when the two drugs were given simultaneously.

In an attempt to reduce the number of injections of licheniformin and yet maintain therapeutic action, an orienting experiment (Exp. 7) was made in which comparatively large doses were given, but at infrequent intervals. Six mice, infected by inhalation of 10^5 bacilli per mouse, were treated, from one day after infection until the termination of the experiment at 6 weeks, with single subcutaneous injections of 5 mg. on Mondays, Wednesdays and Fridays (i.e. 15 mg. weekly). Another group of 12 mice, similarly infected, was treated with sulphetrone, 3 per cent in the diet, plus 1·5 mg. licheniformin injected daily in the usual two unequal divided doses, and a further group of 12 mice was treated with streptomycin, 3 mg. (base) daily. The main control group was composed of 12 mice. The group percentage scores of lung lesions in the survivors (all animals in the experiment with the exception of one in the sulphetrone-licheniformin group) were as follows: Graded controls (10^3 bacilli), 29; graded controls (10^4 bacilli), 52; main controls (10^5 bacilli), 69; streptomycin, 3 mg. daily (10^5 bacilli), 14; sulphetrone, 3 per cent, plus licheniformin, 1·5 mg. daily (10^5 bacilli), 17; licheniformin, 5 mg. thrice weekly (10^5 bacilli), 17. Thus suppression of the disease after large doses of licheniformin, given thrice weekly, was as marked as in the group treated with sulphetrone together with licheniformin at a lower dose, twice daily.

Although, as already mentioned, there were no macroscopic lesions in some of the licheniformin-treated and streptomycin-treated mice in the above series of experiments, cultures from apparently negative lungs yielded scanty to moderate growth of tubercle bacilli.

DISCUSSION.

Chemical Nature of Licheniformin.

Little can yet be said about the chemical structure of licheniformin. The unfavourable physical properties of the material have led to the failure of attempts at further purification by ordinary methods. It is, therefore, not possible to come to any conclusion as to the homogeneity of the active material, in particular as to whether the toxicity is inherent in the antibiotic, or due to an impurity, and whether "licheniformin" is a single active substance or a group of antibiotics like "penicillin." The uniformity of successive batches of product suggests homogeneity, but would be expected as a result of the uniform treatment adopted in the process of concentration, and any conclusion must await the application of new methods of fractionation.

The applicability to licheniformin of a process of extraction closely resembling that used for streptomycin, coupled with the basic character, suggested a relation between the two compounds, but neither in biological nor in chemical properties so far investigated does further evidence of any connection appear. The material has the character of a salt of a base, with an equivalent weight of about 320, and with between 4 and 5 atoms of nitrogen in one equivalent. The strength and persistence of the Sakaguchi reaction suggests that the guanidino group is present in the active molecule. A notable point is the high proportion of oxygen (*c.* 26 per cent) in the material. Preliminary tests indicate the absence of ketonic or aldehydic oxygen.

Methods of Assessment of Chemotherapeutic Agents in Tuberculous Infection.

The adoption here of the inhalation method of infecting mice with *M. tuberculosis* merits some discussion, particularly as it may prove generally useful as a "screening" test for chemotherapeutic agents in this infection. Following the method previously worked out by one of us (Glover, 1944), mice are infected by inhaling bovine tubercle bacilli in the form of a mist of fine particles, which contains approximately known concentrations of organisms. The assessment of results is based upon the number and distribution of macroscopic lung lesions present at the deliberate termination of the experiment at 4–6 weeks, and not upon survival rates. At this stage, although the peak of progression has been reached, the animals are in good general condition. The degree of pulmonary involvement is roughly proportional to the number of organisms inhaled. The results are highly reproducible and the "score" of lesions of individual mice in a group has a narrow scatter, so that the number of mice in a group can be small.

The route of infection in experimental tuberculosis in mice has been mainly intravenous (Nitti and Jouin, 1942; Martin, 1946; Youmans and McCarter, 1945; Youmans and Williston, 1946; Dubos, 1947). Infection by inhalation has the advantage that lesions are confined to the lungs, whereas with the intravenous route they may occur in other parts of the body and difficulty arises in assessing their number and severity. Furthermore, the distribution of foci in the lungs tends to be less variable in the inhalation method.

One unequivocal advantage of the mouse over the guinea-pig for chemotherapeutic tests lies in the smaller amount of drug required and the shorter period of observation necessary. It is, however, a matter of speculation which animal may yield results more indicative of therapeutic value in man. The guinea-pig's high degree of sensitivity, which makes experimental infection with large numbers of fully virulent bacilli inadvisable for fear of evoking a fulminating disease that might mask the action of an agent having moderate bacteriostatic properties, contrasts with the mouse's high intrinsic resistance, which, after inhalation of relatively large numbers of bacilli, results in considerable pulmonary involvement without death. It should be noted also that there are specific differences in response to anti-tuberculous agents by mice and guinea-pigs, for streptomycin is suppressive in both species (Youmans *et al.*, 1945, 1946; Smith and McClosky, 1945; Feldman, Hinshaw and Mann, 1945; Dickinson, 1947), whilst sulphone compounds have marked activity in guinea-pigs (Feldman, Hinshaw and Mann, 1944; Brownlee, 1948), but not in mice (Gunn and Youmans, 1945). The present investigations confirm the reported effectiveness of streptomycin in mice, and the apparent inactivity of sulphones alone.

The Activity of Licheniformin, alone and with Sulphetrone, against Tuberculous Infection.

The action of licheniformin in suppressing experimental tuberculosis in mice is clearly shown by the results given above and summarized in Table IX and Fig. 2. However, administration over the period of the tests leads to subacute or chronic renal changes, and this effect makes it inadvisable to apply directly the material now available to treatment of human tuberculosis. Nevertheless, in the present primitive state of the chemical investigation, the possibility is not excluded that a purer material with a more favourable therapeutic index may be prepared. It

also remains to be seen whether practical use can be made of administration in less frequent but larger doses, or, in the light of the results discussed below, of combination with other agents.

Evidence had already been obtained (Smith and McClosky, 1945; Smith, McClosky and Emmart, 1946; Smith, McClosky, Jackson and Bauer, 1947; Callomon, Kolmer, Rule and Paul, 1946) of the synergistic effect of streptomycin and certain sulphones upon experimental tuberculosis in various species. Brownlee (1948) demonstrated an effect of this type in the guinea-pig with streptomycin and sulphetrone (tetrasodium 4 : 4′-bis-(γ-phenyl-*n*-propylamino)diphenylsulphone αγα′γ′-tetrasulphonate), which is of low toxicity. We have found no effect with sulphetrone, at doses below the toxic level, on tuberculous infection in mice, but when moderate doses of licheniformin were also given, the effect was considerably greater than that of the same dosage of licheniformin alone. The existence of a synergistic rather than a simply additive effect seems to be certain in this instance, unlike some other cases in which independent activity of two drugs makes the recognition of true synergy difficult.

The combined action of streptomycin and licheniformin did not appear to be more than simply additive. The use of these two antibiotics might, however, have application in tuberculosis in man, where, as has been shown, resistance to streptomycin may develop after prolonged treatment. The sensitiveness of streptomycin-resistant strains of *M. tuberculosis* to licheniformin is now being investigated.

Should licheniformin, whether alone or with other agents, not prove to be of practical use, it is yet clear that the constitution of one of the few compounds able to suppress experimental tuberculous infection is of the greatest interest.

SUMMARY.

1. Methods are described for the culture on simple synthetic media of *Bacillus licheniformis* on a small or larger scale for the production of the antibiotic licheniformin.

2. Licheniformin is extracted from culture media by adsorption on carbon, elution, and conversion into the picrate and hydrochloride.

3. Licheniformin is a base, the hydrochloride of which is an amorphous material, very soluble in water, and in solution fairly stable to heat-treatment. The hydrochloride has the approximate composition C, 40; H, 7·5; N, 17·4; Cl, 9 per cent. Other derivatives have been prepared; the helianthate and reineckate are crystalline. The chemical and biological properties of licheniformin distinguish it from other antibiotics derived from the *B. subtilis* group.

4. Licheniformin has bacteriostatic activity against a number of species, including certain corynebacteria, staphylococci, streptococci, pasteurellae, brucellae, mycobacteria and *B. anthracis*; the growth of strains of *M. tuberculosis* tested is inhibited by concentrations of $1 : 1{\cdot}6 \times 10^5$ to $1 : 6{\cdot}4 \times 10^5$. Activity *in vitro* is found to decrease with increasing size of inoculum of the test organism and with increasing acidity of the medium; it is little affected by the presence of serum.

5. Licheniformin-like activity has been found in the culture fluid of each of 11 strains of *B. licheniformis* tested, but in none of 70 strains of other species of aerobic spore-forming bacilli when grown under the same cultural conditions.

6. Licheniformin has moderately toxic effects in mice, injection of high doses causing death, and repeated lower doses causing damage to the kidneys. The effect on blood, pharmacological reactions, and the level in blood after subcutaneous administration have been investigated.

7. Licheniformin has some protective effect against infections by *Staph. pyogenes*, *Str. pyogenes* and *B. anthracis* in mice. It has a considerable suppressive effect on tuberculous infection produced in mice by an inhalation method; parallel tests have been carried out with streptomycin. In simultaneous treatment with licheniformin and sulphetrone the two agents have a synergistic effect in tuberculous infection.

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DEHYDROGENASE ACTIVITY IN VIRUS INFECTIONS.

D. J. BAUER.

From the Wellcome Laboratories of Tropical Medicine.

Received for publication November 11, 1947.

WHILE much work has been carried out upon the enzymic activity of neoplastic tissues, comparatively little is known about the biochemistry of virus infections. Information of this kind is essential for planning a rational approach to a chemotherapeutic attack on virus diseases, in that it may be expected to throw light upon the metabolic processes which are concerned in virus multiplication. While it has been reported that the elementary bodies of vaccinia contain phosphatase and catalase (Macfarlane and Salaman, 1938), these enzymes occur in abundance in most tissues, and it is quite likely that they are present in elementary body suspensions as impurities. From the evidence so far available it seems probable that virus particles have no enzyme activity, or at best a very restricted one. Enzymes required in virus metabolism must therefore be provided by the host; a study of the occurrence of enzymes in normal tissues which are available habitats for virus growth should indicate those processes which are available to the virus for its own metabolism, and an investigation of the same nature during the course of a virus infection should show what modifications are imposed upon the normal cellular economy by the demands of the parasite.

The association of xanthine oxidase with virus infections has already been briefly reported (Bauer, 1947); in the present paper this work is presented in greater detail in relation to a general investigation of the dehydrogenase activity of virus-containing tissues. Xanthine oxidase has also been studied in relation to nucleic acid metabolism in virus infections, and this aspect of virus metabolism will be described in a separate communication.

METHODS.

Viruses.

Experiments were carried out with the neurotropic strain of yellow fever virus and the viruses of lymphocytic choriomeningitis and lymphogranuloma inguinale. These viruses were chosen because they are serologically unrelated and differ markedly in particle size, and might therefore be expected to show

metabolic differences. They will all proliferate in mouse brain, and this tissue was therefore chosen for study. The viruses were maintained by serial intracerebral passage; all the tissue preparations used contained virus at constant maximum titre (yellow fever -10^7; lymphocytic choriomeningitis -10^7; lymphogranuloma inguinale -10^3).

Brain suspensions were prepared as follows. The brain was removed, ground in a mortar and suspended in 5 ml. distilled water. This was centrifuged at low speed to throw down tissue debris; the supernatant fluid was then decanted carefully to avoid admixture with the sediment.

Enzyme determinations.

All experiments were carried out by the Thunberg technique, using 1 ml. of brain suspension, 0·5 ml. of 1/20,000 methylene blue solution, and 1 ml. of buffer solution, adding 0·25 or 0·5 ml. of substrate. Since normal tissue suspensions decolorize methylene blue, a blank tube was always set up containing buffer solution in place of substrate. The tubes were placed in a 37° C. bath and the time for decolorization was noted. A numerical value for the enzyme activity was obtained from the expression $60\left(\frac{I}{T}-\frac{I}{B}\right)$, in which T and B represent the time in minutes necessary for decolorization of the test and blank respectively. The enzymes investigated are listed in Table I, together with the pH and amount of substrate used.

TABLE I.

Enzyme.	pH.	Substrate.	
		Volume ml.	Molarity.
Xanthine oxidase	10·0	0·25	0·0066
Succinic dehydrogenase	7·0	0·5	0·05
Lactic dehydrogenase	7·0	0·5	0·05
α-glycerophosphate dehydrogenase	7·0	0·5	0·05
Pyruvic dehydrogenase	7·0	0·5	0·05
Malic dehydrogenase	7·0	0·5	0·025

RESULTS.

Extracts of normal tissues will decolorize methylene blue *in vacuo* in the absence of added substrate. This is brought about by enzyme-substrate reactions occurring in the tissue extract and by the presence of various substances which reduce methylene blue directly; for the sake of convenience the effect is referred to here as the total dehydrogenase activity, although it probably includes reactions which are non-enzymatic in nature.

The total dehydrogenase activity of suspensions of virus-infected brain is shown in Table II. The figures represent the time in minutes required to decolorize methylene blue at pH 10 under the conditions already described.

In the case of all three viruses the total dehydrogenase activity shows a considerable increase over the values observed for normal mousebrain. Further information as to the cause of this increase was obtained by individual study of the activity of the dehydrogenases listed above. The results are shown in Table III.

TABLE II.

Virus.	No. of experiments.	Average.	Range.
Yellow fever	18	41·0	17–60
Lymphocytic choriomeningitis	8	40·0	24–59
Lymphogranuloma inguinale	8	38·4	30–49
Normal mouse brain	20	86·9	55–130

TABLE III.

Substrate.	Normal.	Yellow fever.	Lymphocytic chorio-meningitis.	Lympho-granuloma inguinale.
Xanthine	0·17	1·05	1·47	1·13
Pyruvate	0·00	0·94	0·84	1·43
Succinate	3·46	4·0	4·30	5·07
Lactate	1·16	1·54	1·3	2·2
Malate	0·40	1·02	0·83	0·96
α-glycerophosphate	4·22	3·56	3·52	3·20

In the case of two enzymes, xanthine oxidase and pyruvic dehydrogenase, brains containing virus show a much higher activity than normal brains. No definite evidence of pyruvic dehydrogenase activity could be found in normal brains, but it cannot be concluded that this enzyme is entirely absent, as the Thunberg technique cannot be relied upon for detecting very slight activity. Three of the other four enzymes appear to have a slightly increased activity in virus infection, but this is probably not significant. This can be better appreciated from Fig. 1, which shows the number of experiments carried out and the range of values found; xanthine oxidase and pyruvic dehydrogenase activities show an undoubted increase over normal, whereas with the other enzymes the ranges of normal and infected values mostly overlap. There is also an apparent decrease in α-glycerophosphate dehydrogenase activity, but this is again of doubtful significance.

Virus encephalitis in general is characterized by an infiltration of round cells into the substance of the brain. These cells, which are presumably lymphocytes, contain enzymes, and it is of importance to know to what extent their enzyme activity contributes towards the overall enzyme activity of a brain suspension. This point was investigated by preparing suspensions of cervical and axillary lymph glands of normal mice and testing for enzyme activity with the same techniques as were used in the mouse brain experiments. The results are summarized in Table IV. The figures each represent an average of 6 experiments,

TABLE IV.

Substrate.	Average value.
Xanthine	2·57
Pyruvate	0·67
Succinate	1·25
Lactate	0·38
Malate	0·71
α-glycerophosphate	1·64

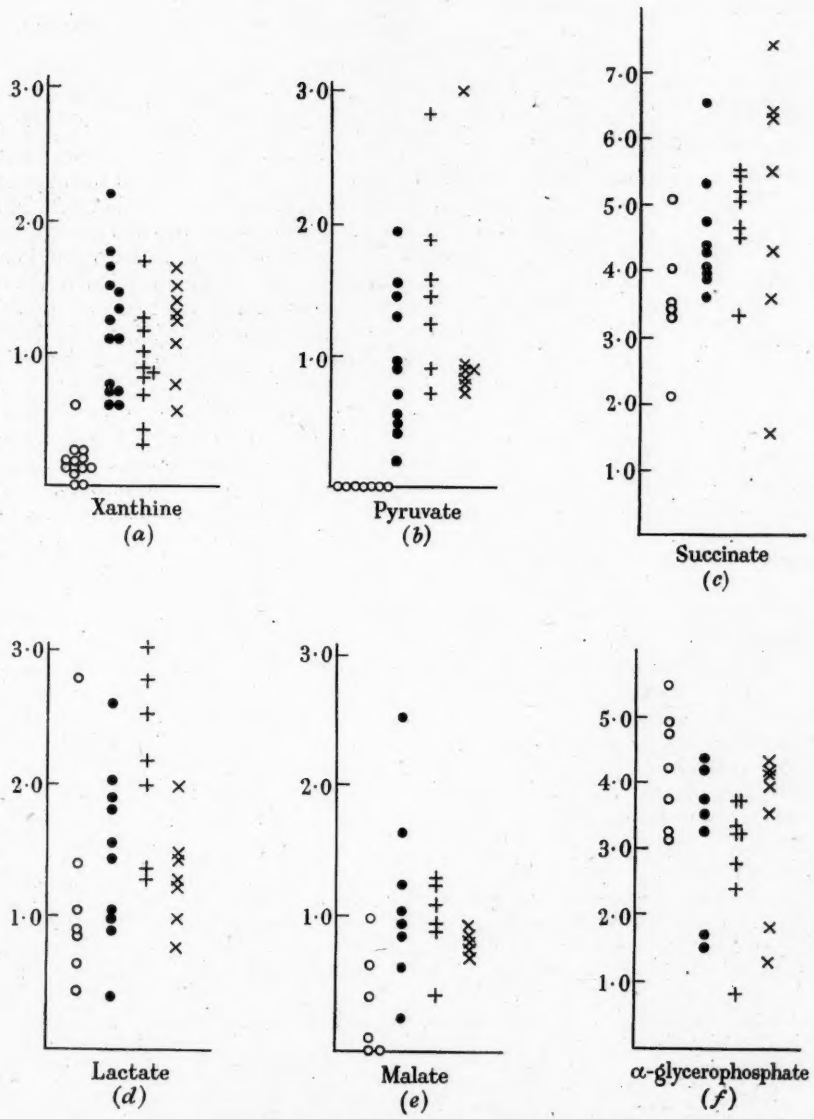

FIG. 1.—Ordinate represents enzyme activity. Vertical scale reduced in (c) and (f).
○ Normal. + Lymphogranuloma inguinale.
● Yellow fever. × Lymphocytic choriomeningitis.

and are directly comparable with the figures already given for the mouse brain suspensions. Their significance will be discussed below.

DISCUSSION.

Of the six enzymes studied, two, namely xanthine oxidase and pyruvic dehydrogenase, are significantly increased in amount during infections with the viruses of yellow fever, lymphocytic choriomeningitis and lymphogranuloma inguinale. The question naturally arises as to whether the increase of enzyme activity is directly concerned with the fact that active multiplication of virus is occurring, or whether it is an incidental finding, due perhaps to the accumulation of inflammatory cells which possess a high enzyme content. In a previous communication (Bauer, 1947) it was shown that the rise in xanthine oxidase activity appears two or three days before any marked infiltration of round cells occurs; it was also shown that the desoxyribonuclease activity of virus-containing brains is not greater than that of normal brains, whereas lymphatic tissue extracts have a very high content of this enzyme (up to 60 times as great as normal mouse brain). These findings indicate that the increase in enzyme activity occurring during a virus infection cannot be accounted for by the accumulation of inflammatory cells. While these undoubtedly increase the overall enzyme content of the tissue this increase is too small to be measurable, and the observed rise in xanthine oxidase and pyruvic dehydrogenase activity must be brought by some other means. A study of Table IV leads to essentially the same conclusion. To produce an increase of xanthine oxidase activity to the level seen in virus infections would require the brain to contain up to half as many lymphocytes as the lymph glands themselves. Study of histological sections shows that the infiltration which occurs does not in any way approach this degree of intensity. Moreover, a hypothetical infiltration of this degree would increase the pyruvic dehydrogenase activity only to 0·3, which is well below the values for virus infections quoted in Table III; it would also cause a rise in α-glycerophosphate dehydrogenase activity of 0·8, whereas reference to Table III shows that no such increase was observed. The figures given in Tables III and IV can in fact only be reconciled by assuming that lymphocytic enzymes do not make any detectable contribution to the overall activity of virus-containing tissues.

As soon as this point is conceded it at once becomes probable that the rise of enzyme content is significantly connected with the fact that virus particles are being actively synthesized, and that the enzymes are in fact required for virus multiplication and accumulate in the infected tissue for that very reason. There are several possible mechanisms by which this might be brought about:

(1) The enzymes might be transported in the blood stream from depots in other organs, and be picked up by the virus particles by virtue of their having a selective affinity for the enzyme molecules.

(2) The virus particles might stimulate the cellular mechanisms which are responsible for the synthesis of enzymes.

(3) The virus particles might themselves bring about the synthesis of enzyme molecules from constituents supplied from the host cells.

(4) There is one further possibility—that the viruses themselves have enzyme activity. This is unlikely, since there is no correlation between amount of enzyme

and amount of virus during the incubation period. In the case of xanthine oxidase and the 17D strain of yellow fever already reported (Bauer, 1947) the following figures were obtained :

Day after infection.	Enzyme activity.	Virus titre.
1	0·25	$10^{1.5}$
4	0·7	10^{4}
7	1·6	10^{6}

In the first four days enzyme activity increases nearly three times while the quantity of virus present increases over 100 times, and a similar lack of correlation is observed from the 4th to the 7th day.

It would be difficult to obtain evidence that enzyme accumulated in the brain from elsewhere in the body. Xanthine oxidase occurs in several organs in the mouse, and it would be necessary to investigate the enzyme balance of the whole animal to determine whether any shift to the brain takes place. This is scarcely a practical proposition when the mouse is used as the experimental animal, and information on this point can be obtained more satisfactorily by investigating virus infection in the chick embryo. A certain amount of preliminary work, which will be reported separately, has already been carried out along these lines ; it is quite practicable to estimate the enzyme content of the whole embryo, or even of the whole egg, and the results so far obtained indicate that the total enzyme content of the embryo is increased during infection with yellow fever virus. This suggests that the rise in enzyme content cannot be accounted for by simple redistribution.

With regard to the second possibility, that virus particles act upon the cellular mechanisms which are responsible for enzyme production, little can be said at present. It is supposed that genes cause the production of protein complexes which pass out into the cytoplasm and there give rise to enzymes, but definite information on this subject is still lacking, and the action of virus particles upon such enzyme precursors cannot at present be the subject of experimental investigation.

The third possibility, that virus particles themselves synthesize enzymes, is worthy of further note. It is accepted as a strong probability that genes exert their influence upon heredity by acting as the initiators of a series of reactions, which culminate in the formation of enzymes which represent the means of expression in reality of the stored potentialities of the genome. It is suggested that this process begins by a condensation of amino-acids against the nucleotide constituents of the gene nucleoproteins, which act as a mould for the assembly of a protein molecule which is eventually transformed into an enzyme. Now viruses and genes exhibit many properties in common, in that they consist largely of nucleoprotein, are self-reduplicating entities, and are capable of profoundly modifying the cellular economy. What is a reasonable supposition in the case of the gene can logically be applied to the case of the virus, and it can be postulated that the virus particles bring about an assemblage of enzyme molecules from constituent parts supplied by the host cell. The virus would then create around itself a focus of biochemical autonomy which would override the normal activities of the cytoplasm. The amount of enzyme formed would, moreover, be limited by the available supply of constituents, and would not be linearly related to virus titre.

The three possibilities outlined above have one feature in common, in that they require that a virus particle should have some special affinity for other substances; they are in no way mutually excluding and could co-exist in actual fact. In the first case there must be an affinity for enzymes sufficient to cause their accumulation in the infected cell. It has already been reported (Loring, 1941) that tobacco mosaic virus forms a reversible combination with ribonuclease, while Hoagland *et al.* (1942) have shown that vaccinia elementary bodies will adsorb certain enzymes from solution. These authors were concerned with the effect of this observation upon the validity of previous reports that elementary bodies possessed enzyme activity, but their work provides evidence of an affinity of the kind postulated above, which might play an essential part in the metabolism of the virus.

The second case resembles the first in that it presupposes some sort of combination between virus particles and the mechanisms responsible for the synthesis of cellular enzymes, while the third possibility requires an affinity to exist between the virus and the constituent parts of an enzyme molecule.

It can be fairly assumed that the fact that a rise of xanthine oxidase and pyruvic dehydrogenase activity occurs during virus infection implies, firstly, that these enzymes are required for virus multiplication, and secondly, that they are present in the normal cell in quantities insufficient for this purpose. The rise in xanthine oxidase activity affords evidence that the virus nucleic acid is built up in part from xanthine, rather than from inosine, adenylic acid, or more complex substances. Pyruvic dehydrogenase occupies a key position at the junction of the protein, fat, and carbohydrate metabolic cycles, and its increase in amount implies that the available enzyme equipment in one of these cycles is inadequate, without, however, affording evidence as to which is implicated.

SUMMARY.

(1) A study has been made of the dehydrogenases present in mouse brain infected with the viruses of yellow fever, lymphocytic choriomeningitis and lymphogranuloma inguinale.

(2) Virus-infected brains show a significant increase in dehydrogenase activity compared with normal brains.

(3) This increase is due, in part at least, to an increase in xanthine oxidase and pyruvic dehydrogenase activity.

(4) It is suggested that these enzymes are required in increased amount because they are essential for virus multiplication, and the mechanism which is responsible for bringing this about is discussed.

Most of the experimental work was carried out by Miss P. H. Jewitt, whose assistance is gratefully acknowledged.

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SURVEY OF PAPERS.

RUBBO has tested the systemic toxicity of 65 acridine derivatives in mice and finds that toxicity, like antibacterial action, is greatest in the highly ionized acridines and least in those that are poorly ionized. The degree of ionization, however, increases antibacterial action more than toxicity, so that there would still appear to be scope for the discovery of new compounds in the acridine series with high antibacterial activity and low toxicity (p. 1).

MATHER, BOWLER, CROOKE AND MORRIS have studied the method of determining plasma volume by injection of Evans Blue with a view to finding the relation between the concentration of dye and time of sampling, so that a correction can be made for the dye that escapes from the plasma during the interval between injection and sampling (p. 12).

EVANS finds that *Cl. welchii* antiserum rich in anticollagenase, but containing no α antitoxin, is able neither to protect guinea-pigs against *Cl. welchii* injection nor enhance the protective properties of α antitoxin. He thus adds further confirmation to his earlier finding that the α antitoxin component of *Cl. welchii* antiserum is of prime importance in the control of *Cl. welchii* infection in guinea-pigs (p. 24).

BORODIN, PHILPOT AND FLOREY describe the purification and some of the chemical and biological properties of an antibacterial substance, tardin, that has been extracted from *Penicillium tardum*. Its properties are such as to make it unlikely that it has therapeutic possibilities (p. 31).

HEATLEY, JENNINGS AND FLOREY find that a strain of the fungus *Stereum hirsutum* liberates during its growth in a fluid medium a precursor which it subsequently converts into an antibacterial substance. This active substance can also be produced by incubating the precursor with the washed mycelium of the fungus or with an extract of mycelium. The two substances have been purified and the precursor has been crystallized, but they do not appear to have any therapeutic potentialities (p. 35).

PURVES AND GRIESBACH report the formation of thyroid adenoma and adenocarcinoma in a large proportion of rats after prolonged administration of the goitrogenic substance thiourea. These experimental tumours are fully as responsive as normal thyroid to fluctuations in thyrotropic stimulation (p. 46).

WILKINS, continuing his investigation into the production of bacteriostatic substances by fungi, describes his findings in a further series of basidiomycetes of the wood-destroying type (p. 53).

WILSON, SMITH, HALE AND SMITH, continuing their studies in staphylocoagulase, find that certain strains of staphylococci differ from most staphylococci in being capable of coagulating guinea-pig plasma. These strains are also more virulent for guinea-pigs than strains which do not coagulate guinea-pig plasma irrespective of their capacity to produce α toxin. The results suggest that coagulase is a major factor in the initiation and development of staphylococcal lesions (p. 57).

SURVEY OF PAPERS.

ALBERT, RUBBO, GOLDACRE AND BALFOUR find that the antibacterial activity of compounds related to oxine (8-hydroxyquinoline) is correlated with chelating power, that is to say, ability to combine with polyvalent metals. They suggest that the active members of this series of compounds function by disorganizing the trace-metal mechanism essential for bacterial growth (p. 69).

UNGAR finds that free penicillin can be detected in the blood-stream of mice, rats and guinea-pigs after the intramuscular injection of the methyl or the benzyl ester of penicillin but not in man or rabbits. This species difference can be attributed to the presence of a penicillin esterase in the former group of animals but absent in the latter. Penicillin esters are thus unlikely to have therapeutic value in man (p. 88).

ARNSTEIN AND COOK show that parasiticin, an antibiotic produced by *Aspergillus parasiticus*, is largely, if not wholly, penicillin-II (p. 94).

HAYES describes a new type of prozone which he has obtained in agglutination tests with human serum and somatic suspensions of *Salm. typhi*. The development of the prozone is determined solely by treatment of the bacterial suspension with heat, and is probably due to the inhibiting effect of absorption by the cell-agglutinin complex of part of the albumen fraction of serum (p. 98).

BATTY AND GLENNY find that the flocculation reaction can be used for determining the total antigen of *Cl. welchii* epsilon toxin both before and after activation by trypsin, provided that the antitoxin has been refined by the pepsin process. Toxicity and combining power can be measured by intravenous injection in mice and by intracutaneous injection in rabbits and guinea-pigs (p. 110).

STONER AND GREEN have confirmed the observation of Quastel and Racker that the saline extracts of ischaemic muscle have greater clot-promoting activity than those prepared from normal muscle. They show that the greater activity of ischaemic muscle is due to thromboplastin, which they consider is normally stored in an inactive form and is mobilized during ischaemia (p. 127).

HARE, MACKENZIE, MCCLELLAND AND CURL find that at least 1 c.c. of concentrated mixed influenza A and B eluate vaccine should be given to give a maximum serological response (p. 141).

ROBSON reports that after the injection of milk fat in 5 cases of sprue there was a fall in total cholesterol and in the free/ester cholesterol ratio, whereas in 5 normal individuals there was a rise in cholesterol and a maintenance of the free/ester cholesterol ratio. He suggests that the failure to absorb fatty acids in sprue may be partially the result of an impairment of fat transport that is mediated by cholesterol (p. 152).

SURVEY OF PAPERS.

McNALLY describes a new series of derivatives of succinic acid which inhibit the growth of *Myco. tuberculosis* and haemolyse red blood cells. The antibacterial and haemolytic activities of these compounds are greatest when the number of carbon atoms in the alkyl substituents is 13 or 14. Both types of activity are reduced by serum albumin (p. 161).

STERNBERGER finds that 25 per cent ethyl alcohol suppresses the antithrombic activity of plasma and describes a simple two-stage method for the determination of prothrombin (p. 168).

BROWN finds that oedematiens antisera flocculate toxin and toxoid equally readily when their antitoxic value is higher *in vivo* than *in vitro*, whereas normal ratio sera flocculate much more readily with toxoid. High ratio sera are therefore more suitable as standard sera for *in vitro* estimations of toxins and toxoids (p. 178).

GREEN AND STONER finds that the lethal effect of an intravenous dose of a fresh saline muscle extract is due to the combined action of adenosine triphosphate and related compounds. The loss of lethal action that occurs in such an extract standing at room temperature is due to enzymic destruction of adenosine triphosphate, and heating at 60° C. for 15 min. inactivates both adenosine triphosphate and thromboplastin (p. 189).

HALE finds that the small colony " G " (gonidial) forms of staphylococci, obtained by exposure of the parent strains to barium chloride or gentian violet, are discontinuous variants. They are much less active in their metabolic activities and less resistant to heat and penicillin than the parent strains (p. 202).

In continuation of work on the production of bacterial inhibitors by modification of essential metabolites, FILDES AND RYDON have studied the effect of methyl substitution in indole and tryptophan. The methyl derivatives are found to exert inhibitory actions, the methyl indoles by competition with indole for the use of the enzyme of which it is the substrate, and the methyl tryptophans by competition with tryptophan. Differences in the potency of the substituents are used as evidence on the nature of the linkage between the metabolites and their enzymes (p. 211).

PATERSON, PIRIE AND STABLEFORTH describe the isolation of an antigenic substance from *Brucella abortus*, its physical and chemical properties. Injection of the aggregated antigen causes a rise in blood agglutinins and protects the animal from infection. Inoculation, two and six weeks before infection, produces more immunity than a single larger dose four weeks before infection. Neither is, however, as efficient as the S. 19 vaccine. Intradermal inoculation of the antigen, especially if disaggregated, produces necrotic lesions in the sensitized animal: intramuscular injection avoids this disadvantage of repeated injections (p. 223).

SURVEY OF PAPERS.

EDWARD has grown louping-ill virus in the chick embryo, obtaining suspensions with L.D.$_{50}$ titres between 10^{-5} and $10^{-7 \cdot 5}$. Infection was fatal in the embryo and afforded a sensitive method for titrating virus. Passage in the egg reduced the ability of the virus to cause encephalitis in sheep inoculated subcutaneously (p. 237).

WILKINS has tested a further 100 species of fungi, all basidiomycetes, for the production of bacteriostatic substances. He compares some of his findings with other workers and discusses the criteria that should be used for indicating the antibacterial status of any given basidiomycete (p. 247).

BOSWELL has made electron micrographs of elementary bodies of the viruses of molluscum contagiosum, fowl-pox and ectromelia. All three viruses appeared to be brick-shaped, although flattened in one direction presumably by drying. There was no evidence of the presence of a cell wall or of multiplication either by budding or binary fission (p. 253).

ELLINGER, KADER and EMMANUELOWA find that ambamide (marfanil) in concentrations of 4 to 40 μg. per ml. stimulates the growth and nicotinamide production of coliform bacteria, but inhibits the growth of *Proteus* and *Streptococcus faecalis* which consume nicotinamide. The administration of ambamide to rats increases the nicotinamide content of the faeces and urine, as has been previously found in man (p. 261).

KENNAWAY and TIPLER find that the concentration of ascorbic acid in hypertrophic human prostates is usually between 50 and 110 μg. per g. (p. 274).

ANDREWES and ELFORD find that the pathogenicity to mice of a suspension of living ectromelia virus can be wholly or largely suppressed by mixing it with a suspension of ectromelia virus that has been inactivated by ultra-violet radiation. They discuss the possibility that living virus, masked in this way, may be present in some inactivated virus vaccines and may even play a part in their efficacy (p. 278).

WUNDERLY and WUHRMANN have studied the effect on turbidity and flocculation tests of adding pure γ-globulin and albumen to normal and hepatitic sera. The Thymol, Cephalin and Cadmium tests give similar results, but the Takata test gives a smaller response. The relative responses to the tests of sera carrying increased γ-globulin or the lipoid-carrying α- and β-globulins is discussed from the point of view of the mechanism of the tests (p. 286).

POLLOCK has found that the growth-promoting power of blood for *H. pertussis* is due largely to its ability to absorb toxic material from the culture—a property shared by purified albumen and charcoal, either of which can replace the blood (p. 295).

SURVEY OF PAPERS.

RIMINGTON, STILLWELL and MAUNSELL have shown that it is possible to obtain from house dust specific antigenic material in a high state of purity giving dermal reactions in dust-sensitive individuals. The specificity of this material appears to reside in an association of amino-acids (p. 309).

STILLWELL, RIMINGTON and MAUNSELL find that there is a general chemical similarity between three polysaccharide products from moulds and the preparations from house dust described in the preceding paper (p. 325).

MAUNSELL, WHETNALL and RIMINGTON find that their crude "dust antigen" in dilutions of 10^{-5} produces skin reactions by intradermal tests in 6 per cent of normal subjects and in 70 per cent of persons with allergic symptoms. Sensitivity to dust and to other inhalants often occurred together, but many patients exhibited sensitivity to the dust antigen but not to any other material tested (p. 331).

HEWITT describes a technique for the rapid serological typing of *C. diphtheriae* by agglutination. Some 50 serological types can be recognized in the gravis, intermedius and mitis groups. The development of the agglutination technique promises to be of value in epidemiological studies and in solving problems of variation and the genesis of strains (p. 338).

HALE and MCILWAIN demonstrate a limited correlation between glutamine requirements and virulence in 9 strains of haemolytic streptococci which they examined. While animal passage increased both virulence and glutamine requirement, *in vitro* subculture did not show a constant correlation between diminished virulence and diminished glutamine requirements. No correlation was established between capsulation and glutamine need (p. 346).

KENNAWAY and TIPLER find that there is an increase in the concentration of ascorbic acid in the liver of rats in pregnancy (p. 351).

LINGGOOD and FENTON show that diphtheria toxin of a titre of 50–70 Lf units can be produced by growing *C. diphtheriae* (PW 8 strain) for 3 days in shaking culture on routine digest medium modified only in-so-far that the concentration of maltose in the medium is increased. Under such conditions tributyl citrate inhibits both growth and toxin production, whereas undecanol in lard oil has no adverse effect. Strips of copper, nickel and even stainless steel under certain conditions inhibit toxin production, while silver, aluminium and its alloys have little or no effect (p. 354).

HOLLÁN finds that urease is present in the mucosa of the fundus of the human stomach. Urease is also found in gastric juice of low acidity, but in gastric juice of normal or high acidity it is absent, or present only in slight traces, because the urease is destroyed by pepsin and hydrochloric acid (p. 365).

EDWARD describes methods for testing the effectiveness of vaccines in protecting against infection with louping-ill (p. 368).

SURVEY OF PAPERS.

LEHMANN, in a survey of 324 Indian soldiers, shows that haemoglobin estimation is a more sensitive test for malnutrition than the determination of serum protein. There is some correlation between low serum protein and anaemia, but this correlation would be expected only in macrocytic anaemia, which is found in people living on a diet lacking in vitamins and valuable protein. whereas anaemia due to lack of iron is not necessarily associated with hypoproteinaemia (p. 377),

BARNES has infected rabbits, guinea-pigs and mice with anthrax by the inhalation of dried spores. He describes the infection which is not a true pulmonary disease but serves to show how a generalized infection may be set up by the introduction of infective particles through the uninjured respiratory tract. He discusses the mode of action of Tergitol, a commercial detergent which enhances the virulence of dried anthrax spores (p. 385).

STUART-HARRIS and MILLER have found great variability in the Hirst agglutination-inhibition test with immune animal sera and pairs of sera from cases of influenza during outbreaks in January, 1947. Mouse-neutralization tests have indicated that one of the newly-isolated viruses is related to the PR8 standard strain of influenza virus and that the neutralizing antibody content of the convalescent sera is not closely related to agglutination-inhibition activity (p. 394).

BAWDEN and CROOK have studied the distribution of virus in leaves infected with potato virus X. By incubating fibrous residues of infected leaves with snail enzyme they obtain as much virus as occurs in the sap, but the sizes of the virus particles vary widely in the different kinds of extracts. They have, however, adduced no conclusive evidence to indicate the size and condition of the virus as it is produced and exists in infected cells (p. 403).

WILKINS, continuing his investigation into the production of antibacterial substances by fungi, gives his results with a fourth 100 of the species of basidiomycetes (p. 416).

CALLOW, GLOVER, D'ARCY HART and HILLS describe methods for the production and extraction of the antibiotic licheniformin from *Bacillus licheniformis*. The chemical and biological properties of licheniformin distinguish it from other antibiotics derived from the *B. subtilis* group of bacteria. In mice it is protective against some bacterial infections and has a suppressive effect on tuberculous infection, but is also moderately toxic (p. 418).

BAUER finds that dehydrogenase activity is increased in mouse brains infected with the viruses of yellow fever, lymphocytic choriomeningitis and lymphogranuloma inguinale. This increase is due, in part at least, to an increase in xanthine oxidase and pyruvic dehydrogenase activity. He suggests that these enzymes are essential for virus multiplication (p. 440).

INDEX TO AUTHORS

ALBERT, A., RUBBO, S. D., GOLDACRE, R. J., and BALFOUR, B. G., the influence of chemical constitution on antibacterial activity. Part III: A study of 8-hydroxyquinoline (oxine) and related compounds, 69

ANDREWES, C. H., and ELFORD, W. J., infectious ectromelia: experiments on interference and immunization, 278

ARNSTEIN, H. R. V., and COOK, A. H., the penicillin produced by *Aspergillus parasiticus*, 94

BALFOUR, B. G., see *Albert, A.*

BARNES, J. M., the development of anthrax following the administration of spores by inhalation, 385

BATTY, I., and GLENNY, A. T., titration of *Cl. welchii* epsilon toxins and antitoxin, 110

BAUER, D. J., dehydrogenase activity in virus infections, 440

BAWDEN, F. C., and CROOK, E. M., some properties of potato virus X in leaf extracts made in different ways, 403

BORODIN, N., PHILPOT, F. J., and FLOREY, H. W., an antibiotic from *Penicillium tardum*, 31

BOSWELL, F. W., electron microscope studies of virus elementary bodies, 253

BOWLER, R. G., see *Mather, K.*

BROWN, A. M., the oedematiens toxin antitoxin reaction: with special attention to the nature of the sera employed, 178

CALLOW, R. K., HART, P. D'ARCY, GLOVER, R. E., and HILLS, G. M., licheniformin, an antibiotic substance from *Bacillus licheniformis* active against *Myobacterium tuberculosis*, 418

COOK, A. H., see *Arnstein, H. R. V.*

CROOK, A. C., see *Mather, K.*

CROOK, E. M., see *Bawden, F. C.*

CURL, M., see *Hare, R.*

EDWARD, D. G. FF., culture of louping-ill virus in the embryonated egg, 237

——— methods for investigating immunization against louping-ill, 368

ELFORD, W. J., see *Andrewes, C. H.*

ELLINGER, P., KADER, M. M. ABDEL, and EMMANUELOWA, A., intestinal bacteria and their production and consumption of nicotinamide as affected *in vitro* by *p*-aminomethylbenzenesulphonamide (ambamide), 261

EMMANUELOWA, A., see *Ellinger, P.*

EVANS, D. G., anticolalgenase in immunity to *Cl. welchii* type A infection, 24

FENTON, E. J., see *Linggood, F. V.*

FILDES, PAUL, and RYDON, H. N., inhibition of growth of *Bact. typhosum* by methyl derivatives of indole and tryptophan, 211

FLOREY, H. W., see *Borodin, N.*

——— see *Heatley, N. G.*

GLENNY, A. T., see *Batty, I.*

GLOVER, R. E., see *Callow, R. K.*

GOLDACRE, R. J., see *Albert, A.*

GREEN, H. N., see *Stoner, H. B.*

——— and STONER, H. B., the nature and mode of action of the intravenous toxic factors in saline extracts of muscle and other tissues, 189

GRIESBACH, W. E., see *Purves, H. D.*

HALE, J. H., see *Smith, Wilson.*

——— studies on staphylococcus mutation: characteristics of the "G" (gonidial) variant and factors concerned in its production, 202

——— and MCILWAIN, H., the behaviour of streptococci of varying virulence towards glutamine, 346

HARE, R., MACKENZIE, D. M., MCCLELLAND, L., and CURL, M., immunization of human beings with red cell eluate and other types of influenza vaccine, 141

HART, P. D'ARCY, see *Callow, R. K.*

HAYES, W., the prozone phenomenon in relation to the agglutinability of *S. typhi*, 98

HEATLEY, N. G., JENNINGS, M. A., and FLOREY, H. W., antibiotics from *Stereum hirsutum*, 35

HEWITT, L. F., serological typing of *C. diphtheriae*, 338

HILLS, G. M., see *Callow, R. K.*,

HOLLÁN, S., gastric urease, 365

JENNINGS, M. A., see *Heatley, N. G.*

KADER, M. M. ABDEL, see *Ellinger, P.*

KENNAWAY, E. L., and TIPLER, M. M., the ascorbic acid content of the hypertrophied human prostate, 274

——— the ascorbic acid content of the liver in pregnant rats, 351

LEHMANN, H., relation between serum protein and haemoglobin in 324 Indian soldiers, 377
LINGGOOD, F. V., and FENTON, E. L., the production of diphtheria toxin by submerged culture in shaking flasks, 354

MACKENZIE, D. M., see *Hare, R.*
MCCLELLAND, L., see *Hare, R.*
MCILWAIN, H., see *Hale, J. H.*
MCNALLY, P. A., the action of monoalkyl and dialkyl succinic acids and the monoesters and monoamido-derivatives of alkyl succinic acids on the growth of *Mycobacterium tuberculosis*, 161
MATHER, K., BOWLER, R. G., CROOKE, A. C., and MORRIS, C. J. O. R., the precision of plasma volume determinations by the Evans Blue method, 12
MAUNSELL, K., see *Rimmington, C.*
——— see *Stillwell, D. E.*
——— WHETNALL, E., and RIMINGTON, C., the allergens of house dust: diagnosis and desensitization of dust-sensitive patients with "crude dust antigen," 331
MILLER, M. H., see *Stuart-Harris, C. H.*
MORRIS, C. J. O. R., see *Mather, K.*

PATERSON, J. S., PIRIE, N. W., and STABLEFORTH, A. W., protective antigens isolated from *Br. abortus*, 223
PHILPOT, F. J., see *Borodin, N.*
PIRIE, N. W., see *Paterson, J. S.*
POLLOCK, M. R., the growth of *H. pertussis* in media without blood, 295
PURVES, H. D., and GRIESBACH, W. E., studies on experimental goitre. VIII: Thyroid tumours in rats treated with thiourea, 46

RIMINGTON, C., STILLWELL, D. E., and MAUNSELL, K., the allergens of house dust: purification and chemical nature of active constituents, 309
——— see *Stillwell, D. E.*
——— see *Maunsell, K.*
ROBSON, J. S., plasma and corpuscular cholesterol in sprue, 152
RUBBO, S. D., the influence of chemical constitution on toxicity. Part I: A general survey of the acridine series, 1
——— see *Albert, A.*
RYDON, H. N., see *Fildes, Paul.*

SMITH, M. M., see *Smith, Wilson.*
SMITH, WILSON, HALE, J. H., and SMITH, M. M., the role of coagulase in staphylococcal infections, 57
STABLEFORTH, A. W., see *Paterson, J. S.*
STERNBERGER, L. A., the stabilization of thrombin in plasma: development of a simple two-stage method for the determination of prothrombin, 168
STILLWELL, D. E., see *Rimington, C.*
——— RIMINGTON, C., and MAUNSELL, K., the allergen(s) of house dust; comparison with products derived from moulds, 325
STONER, H. B., and GREEN, H. N., bodily reactions to trauma. The influence of ischaemia on the clotting factor of muscle, 127
——— see *Green, H. N.*
STUART-HARRIS, C. H., and MILLER, M. H., vagaries of the agglutination-inhibition reaction with newly-isolated strains of influenza virus, 394

TIPLER, M. M., see *Kennaway, E. L.*
——— see *Kennaway, E. L.*

UNGAR, J., the *in vitro* activity of penicillin esters, 88

WHETNALL, E., see *Maunsell, K.*
WILKINS, W. H., investigation into the production of bacteriostatic substances by fungi. Preliminary examination of the sixth 100 species, more basidiomycetes of the wood-destroying type, 53
——— investigation into the production of bacteriostatic substances by fungi. Preliminary examination of the seventh 100 species, all basidiomycetes, 247
——— investigation into the production of antibacterial substances by fungi. Preliminary examination of the eighth 100 species, all basidiomycetes, 416
WUHRMANN, F., see *Wunderly, C.*
WUNDERLY, C., and WUHRMANN, F., the effect of experimental increases in the γ-globulin and albumin content of sera on the response given by turbidity and flocculation tests, 286

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THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

VOL. XXVIII, No. 6. DECEMBER, 1947.

CONTENTS.

LEHMANN, H.—RELATION BETWEEN SERUM PROTEIN AND HAEMOGLOBIN IN 324 INDIAN SOLDIERS 377

BARNES, J. M.—THE DEVELOPMENT OF ANTHRAX FOLLOWING THE ADMINISTRATION OF SPORES BY INHALATION 385

STUART-HARRIS, C. H., AND MILLER, M. H.—VAGARIES OF THE AGGLUTINATION-INHIBITION REACTION WITH NEWLY-ISOLATED STRAINS OF INFLUENZA VIRUS 394

BAWDEN, F. C., AND CROOK, E. M.—SOME PROPERTIES OF POTATO VIRUS X IN LEAF EXTRACTS MADE IN DIFFERENT WAYS 403

WILKINS, W. H.—INVESTIGATION INTO THE PRODUCTION OF ANTIBACTERIAL SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF THE EIGHTH 100 SPECIES, ALL BASIDIOMYCETES 416

CALLOW, R. K., GLOVER, R. E., HART, P. D'ARCY, AND HILLS, G. M.—LICHENIFORMIN, AN ANTIBIOTIC SUBSTANCE FROM *Bacillus licheniformis*, ACTIVE AGAINST *Mycobacterium tuberculosis* 418

BAUER, D. J.—DEHYDROGENASE ACTIVITY IN VIRUS INFECTIONS 440

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